NEUROTROPHIC FACTOR RECEPTORS

1. Field of the Invention

The present invention relates to receptors for neurotrophic factors. In particular, the invention relates to receptors for glial cell line-derived neurotrophic factor (GDNF) and neurturin and provides nucleic acid and amino acid sequences encoding the receptors. The present invention also relates to therapeutic techniques for the treatment of neurotrophic factors-responsive conditions.

2. Background of the Invention

Glial Cell line-Derived Neurotrophic Factor

Glial cell line-derived neurotrophic factor (GDNF) was initially isolated and cloned from rat B49 cells as a potent neurotrophic factor that enhances survival of midbrain dopaminergic neurons (Lin et al., Science, 260, 1130-1132, 1993). Recent studies have indicated that this molecule exhibits a variety of other biological activities, having effects on several types of neurons from both the central and peripheral nervous systems. In the central nervous system (CNS), GDNF has been shown to prevent the axotomy-induced death of mammalian facial and spinal cord motor neurons (Li et al., Proceedings Of The National Academy Of Sciences, U.S.A., 92, 9771-9775, 1995; Oppenheim et al., Nature, 373, 344-346, 1995; Yan et al., Nature, 373, 341-344, 1995; Henderson et al., Science, 266, 1062-1064, 1994; Zurn et al., Neuroreport, 6, 113-118, 1994), and to rescue developing avian motor neurons from natural programmed cell death (Oppenheim et al., 1995 supra). Local administration of GDNF has been shown to protect nigral dopaminergic neurons from axotomy-induced (Kearns and Gash, Brain Research, 672, 104-111, 1995; Beck et al., Nature, 373, 339-341, 1995) or neurotoxin-induced degeneration (Sauer et al., Proceedings Of The National Academy Of Sciences U.S.A., 92, 8935-8939, 1995; Tomac et al., Nature, 373, 335-339, 1995). In addition, local administration of GDNF has been shown to induce sprouting from dopaminergic neurons, increase levels of dopamine, noradrenaline, and serotonin, and improve motor behavior (Tomac et al., 1995 supra).

More recently, GDNF has been reported to be a potential trophic factor for brain noradrenergic neurons and Purkinje cells. Grafting of fibroblasts ectopically expressing GDNF prevented 6-hydroxydopamine-induced degeneration and promoted the phenotype of adult noradrenergic neurons in vivo (Arenas et al., Neuron, 15,

1465-1473, 1995), while exogeneously applied GDNF effectively promoted survival and morphological differentiation of embryonic Purkinje cells in vitro (Mount et al., Proceedings Of The National Academy Of Sciences U.S.A., 92, 9092-9096, 1995). In the peripheral nervous system, GDNF has been shown to promote the survival of neurons in nodose, ciliary, and sympathetic ganglia, as well as small populations of embryonic sensory neurons in dorsal root ganglia (DRG) and trigeminal ganglia (Trupp et al., Journal Of Cell Biology, 130, 137-148, 1995; Ebendal et al., Journal Of Neuroscience Research, 40, 276-284, 1995; Oppenheim et al., 1995 supra; Yan et al., 1995 supra; Henderson et al., 1994 supra). GDNF has also been reported to enhance the expression of vasoactive intestinal peptide and preprotachykinin-A mRNA in cultured superior cervical ganglion (SCG) neurons, and thus effects the phenotype of SCG neurons and induces bundle-like sprouting (Trupp et al., 1995 supra).

Expression of GDNF has been observed in a number of different cell types and structures of the nervous system. In the CNS, GDNF mRNA expression has been observed by reverse transcriptase polymerase chain reaction (RT-PCR) in both developing and adult rat striatum, the major target of nigral dopaminergic innervation, and widely in other regions, including hippocampus, cortex, thalamus, septum, cerebellum, spinal cord, and medulla oblongata (Arenas et al., supra 1995; Poulsen et al., Neuron, 13, 1245-1252, 1994; Springer et al., Experimental Neurology, 127, 167-170, 1994; Stroemberg et al., Experimental Neurology, 124, 401-412, 1993; Schaar et al., Experimental Neurology, 124, 368-371, 1993). In human, GDNF transcripts have also been detected in striatum, with highest level in the caudate and lower levels in the putamen. Detectable levels are also found in hippocampus, cortex, and spinal cord, but not in cerebellum (Schaar et al., Experimental Neurology, 130, 387-393, 1994; Springer et al., 1994 supra). In the periphery, GDNF mRNA expression has been reported in DRG and SCG of postnatal day 1 rats, sciatic nerve, and primary cultures of neonatal Schwann cells (Trupp et al., 1995 supra; Hoffer et al., Neuroscience Letters, 182, 107-111, 1994; Henderson et al., 1994 supra; Springer et al., 1994 supra). In addition, recent studies have shown that GDNF transcripts are also widely expressed in peripheral non-neuronal organs, including postnatal testis and kidney, embryonic whisker pad, stomach, and skin. Expression can be detected at lower levels in embryonic muscle, adrenal gland and limb bud, and in postnatal lung, liver and ovary (Trupp et al., 1995 supra; Henderson et al., 1994 supra). So far, however, the biological significance of the non-neuronal expression of GDNF is not clear.

A neurotrophic factor refered to as "neurturin" is described in Nature 384(5):467-470, 1996. Detailed descriptions of the preparation and characterization of

GDNF protein products may be found in U.S. Patent Application No. 08/182,183 filed May 23, 1994, now abandoned, and its parent applications (also see PCT/US92/07888, WO 93/06116 filed September 17, 1992 and European Patent Application No. 92921022.7, Publication No. EP 610 254) the disclosures of which are hereby incorporated by reference. Additional GDNF protein products are described in ~~pending~~ now U.S. Patent 6,184,200, U.S. Patent Application No. 08/535,681 filed September 28, 1995, the disclosure of which is hereby incorporated by reference. As used herein, the term "GDNF protein product" includes biologically active synthetic or recombinant GDNF proteins and analogs, as well as chemically modified derivatives thereof. GDNF analogs include deletion variants such as truncated GDNF proteins, as well as insertion and substitution variants of GDNF. Also included are GDNF proteins that are substantially homologous to the human GDNF protein.

GDNF Therapy

GDNF therapy is helpful in the treatment of nerve damage caused by conditions that compromise the survival and/or proper function of one or more types of nerve cells. Such nerve damage may occur from a wide variety of different causes. Nerve damage may occur to one or more types of nerve cells by: (1) physical injury, which causes the degeneration of the axonal processes and/or nerve cell bodies near the site of injury; (2) temporary or permanent cessation of blood flow to parts of the nervous system, as in stroke; (3) intentional or accidental exposure to neurotoxins, such as chemotherapeutic agents (e.g., cisplatinum) for the treatment of cancer or dideoxycytidine (ddC) for the treatment of AIDS; (4) chronic metabolic diseases, such as diabetes or renal dysfunction; or (5) neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS), which result from the degeneration of specific neuronal populations.

Several studies indicate that GDNF therapy is particularly helpful in the treatment of neurodegenerative conditions such as the degeneration of the dopaminergic neurons of the substantia nigra in Parkinson's disease. The only current treatments for Parkinson's disease are palliative, aiming at increasing dopamine levels in the striatum. The expected impact of GDNF therapy is not simply to produce an increase in the dopaminergic neurotransmission at the dopaminergic nerve terminals in the striatum (which will result in a relief of the symptoms), but also to slow down, or even stop, the progression of the degenerative processes and to repair the damaged nigrostriatal pathway and restore its function. GDNF may also be used in treating other forms of damage to or improper function of dopaminergic nerve cells in human patients. Such damage or malfunction may occur in schizophrenia and other forms of

psychosis. The only current treatments for such conditions are symptomatic and require drugs which act upon dopamine receptors or dopamine uptake sites, consistent with the view that the improper functioning of the dopaminergic neurons which innervate these receptor-bearing neuronal populations may be involved in the disease process.

Receptors

A number of receptors which mediate binding and response to protein factors have been characterized and molecularly cloned, including receptors for insulin, platelet derived growth factor, epidermal growth factor and its relatives, the fibroblast growth factors, various interleukins, hematopoietic growth factors and ciliary neurotrophic factor (U.S. 5,426,177). Study results indicate that some receptors can bind to multiple (related) growth factors, while in other cases the same factor can bind and activate multiple (related) receptors (e.g., Lupu et al., Science, 249:1552-1555, 1990; Dionne et al., EMBO J., 9:2685-2692, 1990; Miki et al., Science, 251:72-75, 1991). Most receptors can broadly be characterized as having an extracellular portion or domain responsible for specifically binding a protein factor, a transmembrane domain which spans the cell membrane, and an intracellular domain that is often involved in initiating signal transduction upon binding of the protein factor to the receptor's extracellular portion. Although many receptors are comprised of a single polypeptide chain, other receptors apparently require two or more separate subunits in order to bind to their protein factor with high-affinity and to allow functional response following binding (e.g., Hempstead et al., Science, 243:373-375, 1989; Hibi et al., Cell, 63:1149-1157, 1990).

The extracellular and intracellular portions of a given receptor may share common structural motifs with the corresponding regions of other receptors, suggesting evolutionary and functional relationships between different receptors. These relationships can often be quite distant and may simply reflect the repeated use of certain general domain structures. For example, a variety of different receptors that bind unrelated factors make use of "immunoglobulin" domains in their extracellular portions, while other receptors utilize "cytokine receptor" domains in their factor-binding regions (e.g., Akira et al., The FASEB J., 4:2860-2867, 1990). A large number of receptors with distinct extracellular binding domains (which thus bind different factors) contain related intracytoplasmic domains encoding tyrosine-specific protein kinases that are activated in response to factor binding (e.g., Ullrich and Schlessinger, Cell, 61:203-212, 1990). The mechanisms by which factor-binding "activates" the signal transduction process is poorly understood, even in the case of

receptor tyrosine kinases. For other receptors, in which the intracellular domain encodes a domain of unknown function or in which the binding component associates with a second protein of unknown function (e.g., Hibi et al., Cell, 63:1149-1157, 1990), activation of signal transduction is not well characterized.

The mode of action of GDNF in vivo is not clearly elucidated in the art, in part due to the absence of information on a receptor for GDNF. Two groups have independently found that striatum injected [^{125}I]-labeled GDNF can be retrogradely transported by dopaminergic neurons in the substantia nigra (Tomac et al., Proceedings Of The National Academy Of Sciences Of The United States Of America. 92, 8274-8278, 1995; Yan et al., 1995 supra). Retrograde transport of [^{125}I]-GDNF by spinal cord motor neurons, DRG sensory neurons and neurons in the B layer of retina ganglia was also been observed. These retrograde transport phenomena can all be specifically inhibited by 100-fold or higher concentrations of unlabeled GDNF, suggesting a saturable, receptor-mediated transport process. In vitro, recombinant GDNF has been shown to enhance the survival and promote dopamine uptake of cultured dopaminergic neurons at very low concentrations. The observed half-maximal effective concentration (EC_{50}) of GDNF on these neurons is 0.2 to 1.6 pM (Lin et al., 1993 supra). GDNF has also been shown to support the survival of dissociated motor neurons at low concentrations. The reported EC_{50} of GDNF on motor neurons, in a 5 to 10 fM range, is even lower than that on dopaminergic neurons (Henderson et al., 1994 supra).

Taken together, these observations indicate that receptor(s) for GDNF expressed in these cells have very high ligand binding affinities. Similar to members of the TGF-ß family, the widely diversified tissue distribution and varied biological function of GDNF on different populations of cells suggest that different types of receptor(s) for GDNF or receptor complexes may exist. Saturation steady-state and competitive binding of [^{125}I]-GDNF to E10 chick sympathetic neurons has shown that these neurons express GDNF binding sites differing from those observed in dopaminergic and motor neurons. The half maximal saturation concentration and the half-maximal inhibition concentration of GDNF on these binding sites is in the range of 1 to 5 nM (Trupp et al., 1995 supra). Similarly, the EC_{50} of GDNF in supporting the survival of sympathetic neurons from P1 rat SCG has also been reported to be in the nanomolar range (Trupp et al., 1995 supra).

To better understand the mechanism by which GDNF activates signal transduction to exert its affects on cells, it would be beneficial to identify the receptor(s) which mediate binding and response to this protein factor. It would also be beneficial for GDNF therapy to identify and make possible the production of accessory

molecules which provide for or enhance GDNF signal transduction. Moreover, the identification of a protein receptor for GDNF would provide powerful applications in diagnostic uses, for example, as an aid in determining if individuals would benefit from GDNF protein therapy. Furthermore, the protein receptor for GDNF could be a key component in an assay for identifying additional molecules which bind to the receptor and result in desired biological activity.

SUMMARY OF THE INVENTION

The present invention provides nucleic acid sequences which encode neurotrophic factor receptor proteins having amino acid sequences as depicted in the Figures as well as biologically equivalent analogs. The neurotrophic factor receptor protein and protein products of the present invention are designated herein as glial cell line-derived neurotrophic factor receptor (GDNFR) protein and protein products. Particular receptor proteins refered to herein include GDNFR-α, and glial cell line-derived neurotrophic factor receptor-α-related receptor proteins 2 and 3 (GRR2 and GRR3). The novel proteins are functionally characterized by the ability to bind GDNF and/or neurturin specifically, and to act as part of a molecular complex which mediates or enhances the signal transduction affects of GDNF and/or neurturin. GDNFR protein products are typically provided as a soluble receptor protein and in a substantially purified form.

In one aspect, the present invention provides for the production of GDNFR protein products by recombinant genetic engineering techniques. In an alternative embodiment, the GDNFR proteins are synthesized by chemical techniques, or a combination of the recombinant and chemical techniques.

In another aspect of the present invention, the GDNFR proteins may be made in glycosylated or non-glycosylated forms. Derivatives of GDNFR protein typically involve attaching the GDNFR protein to a water soluble polymer. For example, the GDNFR protein may be conjugated to one or more polyethylene glycol molecules to decrease the precipitation of the GDNFR protein product in an aqueous environment.

Yet another aspect of the present invention includes the various polynucleotides encoding GDNFR proteins. These nucleic acid sequences are used in the expression of GDNFR in a eukaryotic or prokaryotic host cell, wherein the expression product or a derivative thereof is characterized by the ability to bind to GDNF and thereby form a complex capable of mediating GDNF activity, such as increasing dopamine uptake by dopaminergic cells. The polynucleotides may also be used in cell therapy or gene

therapy applications. Suitable nucleic acid sequences include those specifically depicted in the Figures as well as degenerate sequences, naturally occurring allelic variations and modified sequences based on the present invention.

Exemplary nucleic acid sequences include sequences encoding a neurotrophic factor receptor protein comprising an amino acid sequence as depicted in the Figures capable of complexing with glial cell line-derived neurotrophic factor (GDNF) and/or neurturin and mediating cell response to GDNF and/or neurturin, and biologically equivalent analogs thereof. Such sequences include: (a) a sequence set forth in Figure 1 (SEQ ID NO. 1) comprising nucleotides encoding Met^{1} through Ser^{465} or Figure 3 (SEQ ID NO. 3) comprising nucleotides encoding Met^{1} through Ser^{468} encoding a neurotrophic factor receptor (GDNFR-α) capable of complexing with glial cell line-derived neurotrophic factor (GDNF) and mediating cell response to GDNF, as well as GRR2 and GRR3; (b) a nucleic acid sequence which (1) hybridizes to a complementary sequence of (a) and (2) encodes an amino acid sequence with GDNFR activity; and (c) a nucleic acid sequence which but for the degeneracy of the genetic code would hybridize to a complementary sequence of (a) and (2) encodes an amino acid sequence with GDNFR activity. Also disclosed herein are vectors such nucleic acid sequences wherein the sequences typically are operatively linked to one or more operational elements capable of effecting the amplification or expression of the nucleic acid sequence. Host cells containing such vectors are also contemplated. Typically, the host cell is selected from mammalian cells and bacterial cells, such as a COS-7 cell or E. coli, respectively.

A further aspect of the present invention involves vectors containing the polynucleotides encoding GDNFR proteins operatively linked to amplification and/or expression control sequences. Both prokaryotic and eukaryotic host cells may be stably transformed or transfected with such vectors to express GDNFR proteins. The present invention further includes the recombinant production of a GDNFR protein wherein such transformed or transfected host cells are grown in a suitable nutrient medium, and the GDNFR protein expressed by the cells is, optionally, isolated from the host cells and/or the nutrient medium. The present invention further includes the use of polynucleotides encoding GDNFR protein and vectors containing such polynucleotides in gene therapy or cell therapy.

The host cell may also be selected for its suitability to human implantation, wherein the implanted cell expresses and secretes a neurotrophic factor receptor of the present invention. The host cell also may be enclosed in a semipermeable membrane suitable for human implantation. The host cell may be transformed or transfected ex vivo. An exemplary device for treating nerve damage involves: (a) a semipermeable

membrane suitable for implantation; and (b) cells encapsulated within the membrane, wherein the cells express and secrete a neurotrophic factor receptor as disclosed herein. The membrane is selected from a material that is permeable to the neurotrophic factor receptor protein but impermeable to materials detrimental to the encapsulated cells.

Methods for the recombinant production of a neurotrophic factor receptor of the present invention are also disclosed. An exemplary method involves: (a) culturing a host cell containing a nucleic acid sequence encoding a GDNFR protein of the present invention, such as an amino acid sequence depicted in the Figures capable of complexing with glial cell line-derived neurotrophic factor and/or neurturin and mediating cell response to GDNF and/or neurturin, or biologically equivalent analogs thereof; (b) maintaining said host cell under conditions suitable for the expression of said neurotrophic factor receptor by said host cell; and (c) optionally, isolating said neurotrophic factor receptor expressed by said host cell. The host cell may be a prokaryotic cell or a eukaryotic cell. If bacterial expression is involved, the method may further include the step of refolding the neurotrophic factor receptor.

The present invention includes an isolated and purified protein comprising an amino acid sequence as depicted in the Figures capable of complexing with glial cell line-derived neurotrophic factor and/or neurturin and mediating cell response to GDNF and/or neurturin, and biologically equivalent analogs thereof. Exemplary analogs include, but are not limited to, proteins comprising the amino acid sequence Ser^{18} through Pro^{446}, Asp^{25} through Leu^{447} and Cys^{29} through Cys^{442} as depicted in Figure 2 (SEQ ID NO:2) as well as proteins comprising the amino acid sequence Met^{17} through Pro^{449} and Cys^{29} through Cys^{443} as depicted in Figure 4 (SEQ ID NO:4). The proteins of the present invention may be glycosylated or non-glycosylated and may be produced by recombinant technology or chemical synthesis. The present invention further includes nucleic acid sequences encoding a receptor protein comprising such amino acid sequences.

Also disclosed herein are pharmaceutical compositions comprising a GDNFR protein of the present invention in combination with a pharmaceutically acceptable carrier. A variety of other formulation materials may be used to facilitate manufacture, storage, handling, delivery and/or efficacy.

Another aspect of the present invention includes the therapeutic use of GDNFR genes and proteins. For example, a circulating or soluble GDNFR protein product may be used alone or in conjunction with GDNF and/or neurturin in treating disease of or injury to the nervous system by enhancing the activity of transmembrane signaling of GDNF and/or neurturin. Thus, the proteins and pharmaceutical compositions of the

present invention may be used in treating improperly functioning dopaminergic nerve cells, Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis. Alternatively, a recombinant GDNFR gene may be inserted in the cells of tissues which would benefit from increased sensitivity to GDNF or neurturin, such as motor neurons in patients suffering from amyotrophic lateral sclerosis. In yet another embodiment, GDNFR may be used to block GDNF or neurturin activity in cases where the GDNF or neurturin activity is thought to be detrimental. The GDNFR protein may be used to verify that observed effects of GDNF or neurturin are due to the GDNFR protein.

In another aspect of the invention, GDNFR probes may be used to identify cells and tissues which are responsive to GDNF or neurturin in normal or diseased states. Alternatively, the probes may be used to detect an aberrancy of GDNFR protein expression in a patient suffering from a GDNF- or neurturin-related disorder.

In a further aspect of the invention, GDNFR probes, including nucleic acid as well as antibody probes, may be used to identify GDNFR-related molecules. For example, the present invention provides for such molecules which form a complex with GDNFR protein and thereby participate in GDNFR protein function. As another example, the present invention provides for receptor molecules which are homologous or cross-reactive antigenically, but not identical to GDNFR-α, GRR2 or GRR3, including consensus sequence molecules as depicted in the Figures.

The present invention also provides for the development of both binding and functional assays for GDNF or neurturin based on the receptor. For example, assay systems for detecting GDNF activity may involve cells which express high levels of GDNFR-α, and which are therefore extremely sensitive to even very low concentrations of GDNF or GDNF-like molecules. Similar assays may involve neurturin and GRR2. In yet another embodiment, soluble GDNFR may be used to bind or detect the presence of GDNF or GDNF-like molecules.

In addition, the present invention provides for experimental model systems for studying the physiological role of GDNF or neurturin. Such systems include assays involving anti-GDNFR antibodies or oligonucleotide probes as well as animal models, such as transgenic animals which express high levels of GDNFR and therefore are hypersensitive to GDNF and/or neurturin or animals derived using embryonic stem cell technology in which the endogenous GDNFR genes were deleted from the genome. An anti-GDNFR antibody will binds a peptide portion of the neurotrophic factor receptor proteins. Antibodies include monoclonal and polyclonal antibodies. Alternatively, immunological tags for which antibodies already exist may be attached to the GDNFR protein to aid in detection. Such tags include but are not limited to Flag

(IBI/Eastman Kodak) and myc sequences. Other tag sequences such as polyhistidine have also been used for detection and purification on metal chelating columns.

Yet another aspect of the present invention involves the use of GDNFRs to identify ligands which activate receptors as described in the following detailed description and examples. Proteins as well as small molecule neurotrophic factor mimetics may be identified and studied following the binding studies described herein.

Additional aspects and advantages of the invention will be apparent to those skilled in the art upon consideration of the following description, which details the practice of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts a nucleic acid sequence (SEQ ID NO:1) encoding human glial cell line-derived neurotrophic factor receptor (GDNFR-α). The amino acid sequence of the full length GDNFR protein is encoded by nucleic acids 540 to 1934.

Figure 2 depicts the amino acid sequence (SEQ ID NO:2) of the full length human GDNFR-α protein.

Figure 3 depicts a nucleic acid sequence (SEQ ID NO:3) encoding rat GDNFR-α. The amino acid sequence of the full length GDNFR-α protein is encoded by nucleic acids 302 to 1705.

Figure 4 depicts the amino acid sequence (SEQ ID NO:4) of the full length rat GDNFR-α protein

Figure 5 depicts the alignment and comparison of portions of GDNFR-α cDNA sequences produced in various clones as well as the consensus sequence for human GDNFR-α.

Figure 6 depicts the identification of Neuro-2A derived cell lines expressing GDNFR-α.

Figure 7A and 7B depict the results of the equilibrium binding of [^{125}I]GDNF to cells expressing GDNFR-α.

Figure 8 depicts the results of the chemical cross-linking of [^{125}I]GDNF to GDNFR-α and Ret Expressed in cells expressing GDNFR-α.

Figure 9 depicts the results of the induction of c-Ret autophosphorylation by GDNF in cells expressing GDNFR-α.

Figure 10 depicts the results of the induction of c-Ret autophosphorylation by GDNF and soluble GDNFR-α.

Figure 11 depicts the results of the blocking of c-Ret autophosphorylation by a Ret-Fc fusion protein.

Figure 12 depicts the results of the induction of c-Ret autophosphorylation by GDNF in motor neurons.

Figure 13 depicts a model for GDNF signaling mediated by GDNFR-α and Ret.

Figure 14 depicts a nucleic acid sequence (SEQ ID NO: 35) encoding human glial cell line-derived neurotrophic factor receptor-α-related protein 2 (GRR2). The amino acid sequence (SEQ ID NO: 36) of the full length GRR2 protein is encoded by nucleic acids ~~1585~~ 1587 to ~~2989~~ 2978.

Figure 15 depicts a nucleic acid sequence (SEQ ID NO: 37) encoding human glial cell line-derived neurotrophic factor receptor-α-related protein 3 (~~GRR3~~ SEQ ID NO: 38).

Figure 16 depicts a nucleic acid sequence (SEQ ID NO: 39) encoding rat glial cell line-derived neurotrophic factor receptor-α-related protein 2 (~~rat GRR2~~ SEQ ID NO: 40).

Figure 17 depicts a nucleic acid sequence (SEQ ID NO: 41) encoding rat glial cell line-derived neurotrophic factor receptor-α-related protein 3 (~~rat GRR3~~ SEQ ID NO: 42).

Figure 18 depicts the alignment and comparison of various human, rat and mouse GDNFR amino acid sequences.

Figure 19 depicts the alignment and comparison of human, rat and mouse GDNFR-α, GRR2 AND GRR3 amino acid sequences and an exemplary consensus

GDNFR sequence (SEQ ID NO:43).

Figure 20 depicts the alignment and comparison of human and rat GDNFR-α and GRR2 peptide sequences.

Figure 21 (Panels A and B) depicts the binding of neurturin and GDNF to LA-N-% and NGR-38 cells. LA-N-5 (Panel A) and NGR-38 (Panel B) cells were incubated with 50 pM of either [^{125}I]NTN or [^{125}I]GDNF in the absence (light gray bars) or presence of unlabeled GDNF (dark gray bars) or neurturin (black bars) at 4°C for two hours.

Figure 22 depicts the results of the chemical cross-linking of neurturin and GDNF to GDNFR-α and GRR2.

Figure 23 depicts the results of neurturin induced *ret* autophosphorylation in NGR-38 cells.

Figure 24 depicts the results of neurturin induced *ret* autophosphorylation in LA-N-5 cells.

Figure 25 (Panels A and B) depicts the results of neurturin and GDNF induced MAP kinase activation in LA-N-5 and NGR-38 cells.

Figure 26 depicts the amino acid sequences of GDNFR-α, GRR2 and GRR3 are aligned and a consensus sequence (SEQ ID NO:44) is shown above the three receptor sequences. Upper case letters in the consensus sequence indicate amino acids that are conserved in all three receptors, lower case letters indicate that two of the three receptors share that amino acid, and dots indicate all three receptors have a different amino acid at that position. Predicted signal peptide sequences are underlined in GDNFR-α and GRR3; no signal peptide is predicted for GRR2. The hydrophobic C-terminal regions of all three putative receptors are underlined. Potential N-glycosylation sites are shown in boldface and sites conserved between two receptors are outlined by boxes.

DETAILED DESCRIPTION OF THE INVENTION

Glial cell line-derived neurotrophic factor (GDNF) is a potent neurotrophic

factor which exhibits a broad spectrum of biological activities on a variety of cell types from both the central and peripheral nervous systems. It is a glycosylated, disulfide-linked dimer which is distantly related (less than 20% homology) to the transforming growth factor-ß (TGF-ß) superfamily. GDNF's ability to enhance the survival of dopaminergic neurons and other neuron populations demonstrates its therapeutic potential for the treatment of Parkinson's disease as well as other forms of nerve damage or malfunction.

The described biological activities of the neurturin neurotrophic factor (Nature 384(5):467-470, 1996) include promoting the survival of nodose ganglia sensory neurons and a small population of dorsal root ganglia sensory neurons, in addition to superior cervical ganglion sympathetic neurons. The activity suggests the possibility of a common or similar signaling pathway. In addition, the biological activities of neurturin may extend to motor neurons and dopaminergic neurons. Thus, neurturin may be useful in the treatment of diseases for which the use of GDNF may be indicated.

The present invention is based upon the discovery of a high affinity receptor first found on the surface of cultured retinal cells from postnatal rats. These receptors possess an estimated GDNF binding affinity comparable to that of the receptors found in dopaminergic and motor neurons; midbrain dopaminergic neurons (Lin et al., 1993 supra; Sauer et al., 1995 supra; Kearns and Gash, 1995 supra; Beck et al., 1995 supra; Tomac et al., 1995a supra), facial and spinal cord motor neurons (Li et al., 1995 supra; Oppenheim et al., 1995 supra; Yan et al., 1995 supra; Zurn et al., 1994 supra; Henderson et al., 1994 supra). The receptor molecule has been named GDNF receptor-alpha (GDNFR-α) since it is the first known component of a receptor system for GDNF. The present invention also provides the first description of the expression cloning and characterization of GDNFR-α protein. Cells modified to express the recombinant receptor bind GDNF with high affinity. Additional receptor proteins include glial cell line-derived neurotrophic factor receptor-α related receptor proteins 2 and 3 (GRR2 and GRR3).

Using a dopamine uptake assay and [^{125}I]-GDNF binding on cultured cells, high affinity receptors to GDNF were detected on the surface of rat photoreceptor cells. As further described in the Examples, the study of photoreceptor cells lead to the isolation of a cDNA clone by expression cloning for GDNFR-α. The nucleic acid sequence for GDNFR-α encodes a protein of 468 amino acids with 31 cysteine residues and three potential N-glycosylation sites. Next, a nucleic acid sequence from the rat cDNA clone was used to isolate its human homolog which was found to be

nearly identical to the rat receptor at the amino acid level. The human GDNFR-α cDNA sequence encodes a protein of 465 amino acids with the positions of all cysteine residues and potential N-glycosylation sites conserved relative to the rat receptor. This high degree of primary sequence conservation indicated an important role for this receptor in the biological function of GDNF.

As discussed above, many receptors have three main domains: an extracellular or cell surface domain responsible for specifically binding a protein factor; a transmembrane domain which spans the cell's membrane; and an intracellular or cytoplasmic domain that is typically involved in initiating signal transduction when a protein factor binds to the extracellular domain. It was determined, however, that GDNFR-α is unrelated in sequence or structural characteristics to any known protein (such as the consensus sequences found in either receptor kinases or cytokine receptors), lacks a cytoplasmic domain, lacks the C-terminal charged residues characteristic of a transmembrane domain and is anchored to the cell membrane by glycosyl-phosphatidylinositol (GPI) linkage, as described in greater detail below. Although the absence of an intracellular catalytic domain precluded a direct role in transmembrane signaling, the high binding affinity and strong evolutionary sequence conservation further suggested that this receptor was important for GDNF function.

Because GDNFR-α lacks a cytoplasmic domain, it was thought that this receptor must act in conjunction with one or more accessory molecules which play a role in transmembrane signaling. It was then discovered that transgenic mice which lack the gene for GDNF die and have no kidneys. Transgenic mice which lack the gene for c-ret proto-oncogene (Schuchardt, et al., Nature, 367, 380-383, 1994) were found to have a similar phenotype. The c-ret proto-oncogene encodes a receptor tyrosine kinase (RTK) whose normal function had not yet been determined. All RTKs have a similar topology: they possess an extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic segment containing the catalytic protein-tyrosine kinase domain. Binding of a ligand leads to the activation of the kinase domain and phosphorylation of specific substrates in the cell that mediate intracellular signaling. The present invention involves the discovery that a soluble form of GDNFR-α may be used to mediate the binding of GDNF to the c-ret proto-oncogene and thereby elicit a cellular response to GDNF as well as modify its cell-type specificity.

Similar species, called "receptor alpha" components, provide ligand binding specificity but do not have the capacity to transduce signal on their own. Such components are found in the ciliary neurotrophic factor (CNTF) and interleukin-6 (IL-6) receptor systems. Like GDNFR-α, and in contrast to IL-6 receptor, CNTF receptor

binds its ligand with high affinity, has a hydrophobic C-terminus, no cytoplasmic domain, and is anchored to the cell membrane by GPI linkage (Davis et al., 1991). In order to mediate signal transduction, CNTF binds first to CNTF receptor, creating a complex which is able to bind gp130. This inactive complex then binds to LIF receptor to form the active signaling complex (Davis, et al., Science, 260, 1805-1807, 1993). As with the present invention, CNTF receptor (the ligand specific binding component) must be present for signaling to occur but it need not be membrane bound (Economides et al., Science, 270, 1351-1353, 1995).

As further described below, the GDNFR protein may be anchored to a cell surface, or it may be provided in a soluble form. In either case, the GDNFR protein forms a ligand complex with GDNF and/or neurturin, and the ligand complex binds to cell surface receptor to effectuate intracellular signaling. Thus, a soluble form of GDNFR protein may be used to potentiate the action of a neurotrophic factor that binds thereto and/or modify its cell-type specificity.

The GDNFR proteins are unrelated to previously known receptors. There are no apparent matches in the GenBank and Washington University-Merck databases for related sequences. An expressed sequence tag (EST) found in the Washington University-Merck EST database shows 75% homology to a small portion of the coding region of GDNFR-α (approximately 340 nucleotides of the 521 nucleotides of sequence generated from the 5' end of the clone). This clone (GenBank accession #H12981) was isolated from an oligo-dT primed human infant brain library and cloned directionally into the Lafmid BA vector (Hillier, L. et al, unpublished data). The 3' end of the #H12981 clone has been sequenced, but it exhibits no homology to any part of GDNFR-α. The appearance of homology between this #H12981 clone and GDNFR-α over a short region, which homology then disappears, suggests that the #H12981 clone represents an unspliced transcript, or cloning artifact rather than a bona fide cDNA transcript.

Thus, the present invention enables the cloning of a GDNFR protein by providing a method for selecting target cells which express GDNFR protein. By providing a means of enriching for GDNFR protein-encoding sequences, the present invention further provides for the purification of GDNFR protein and the direct cloning of GDNFR-encoding DNA. The present description of the GDNFR nucleic acid and amino acid sequences provides the information needed to reproduce these entities as well as a variety of GDNFR analogs. With this information, GDNFR protein products may be isolated or generated by any means known to those skilled in the art. A variety of means for the recombinant or synthetic production of GDNFR protein are disclosed.

As used herein, the term "GDNFR protein product" includes biologically active purified natural, synthetic or recombinant GDNFR-α, GRR2 and GRR3 (jointly referred to as glial cell line derived neurotrophic factor receptors, GDNFR, GDNFR protein), GDNFR analogs (i.e., GDNFR homologs and variants involving insertion, substitution and deletion variations, such as based on the consensus sequences depicted in the Figures), and chemically modified derivatives thereof. GDNFR analogs are substantially homologous to the GDNFR amino acid sequences set forth in the Figures.

The term "biologically active", as used herein, means that the GDNFR protein product demonstrates high affinity binding to GDNF and/or neurturin and mediates or enhances GDNF-induced or neurturin-induced signal transduction. Using the present disclosure, it is well within the ability of those of ordinary skill in the art to determine whether a GDNFR protein analog has substantially the same biological activity as the GDNFR protein products set forth in the Figures.

The term "substantially homologous" amino acid sequence, as used herein, refers to an amino acid sequence sharing a degree of "similarity" or homology to the GDNFR amino acid sequences set forth in the Figures such that the homologous sequence has a biological activity or function similar to that described for these GDNFR amino acid sequences. It will be appreciated by those skilled in the art, that a relatively large number of individual or grouped amino acid residues can be changed, positionally exchanged (e.g.s, reverse ordered or reordered) or deleted entirely in an amino acid sequence without affecting the three dimensional configuration or activity of the molecule. Such modifications are well within the ability of one skilled in the art following the present disclosure. The identification and means of providing such modified sequences are described in greater detail below. It is preferable that the degree of homology of a substantially homologous protein (peptide) is equal to or in excess of 70% (i.e., a range of from 70% to 100% homology). Thus, a preferable "substantially homologous" GDNFR amino acid sequence may have a degree of homology greater than or equal to 70% of the amino acid sequences set forth for GDNFR-α, GRR2, GRR3 and consensus sequences thereof as depicted in the Figures. More preferably the degree of homology may be equal to or in excess of 80% or 85%. Even more preferably it is equal to or in excess of 90%, or most preferably it is equal to or in excess of 95%.

The percentage of homology as described herein is calculated as the percentage of amino acid residues found in one protein sequence which align with identical or similar amino acid residues in the second protein sequence. Thus, in the case of GDNFR protein homology, the degree of sequence homology may be determined by

optimally aligning the amino acid residues of the comparison molecule to those of a reference GDNFR polypeptide, such as depicted in the Figures or those encoded by the nucleic acid sequences depicted in the Figures, to maximize matches of residues between the two sequences. It will be appreciated by those skilled in the art that such alignment may include appropriate conservative residue substitutions and will disregard truncations and internal deletions or insertions of the comparison sequence by introducing gaps as required; see, for example Dayhoff, Atlas of Protein Sequence and Structure Vol. 5, wherein an average of three or four gaps in a length of 100 amino acids may be introduced to assist in alignment (p. 124, National Biochemical Research Foundation, Washington, D.C., 1972; the disclosure of which is hereby incorporated by reference). Once so aligned, the percentage is determined by the number of aligned residues in the comparison polypeptide divided by the total number of residues in the comparison polypeptide. It is further contemplated that the GDNFR protein sequences of the present invention may be used to form a portion of a fusion protein or chimeric protein which has, at least in part, GDNFR protein activity. The alignment and homology of such a protein would be determined using that portion of the fusion protein or chimeric protein which is related to GDNFR protein activity.

The sources of such substantially homologous GDNFR proteins include the GDNFR proteins of other mammals (such as depicted in the Figures) which are expected to have a high degree of homology to the human GDNFR protein. For example, the degree of homology between the rat and human GDNFR-α proteins disclosed herein is about 93%. Substantially homologous GDNFR proteins may be isolated from such mammals by virtue of cross-reactivity with antibodies to the GDNFR amino acid sequences depicted in the Figures. Alternatively, they may be expressed by nucleic acid sequences which are isolated through hybridization with the gene or with segments of the gene encoding the GDNFR proteins or which hybridize to a complementary sequence of the nucleic acid sequences illustrated in the Figures. Suitable hybridization conditions are described in further detail below.

The novel GDNFR protein products are typically isolated and purified to form GDNFR protein products which are substantially free of unwanted substances that would detract from the use of the present polypeptides for an intended purpose. For example, preferred GDNFR protein products may be substantially free from the presence of other human (e.g., non-GDNFR) proteinaceous materials or pathological agents. Preferably, the GDNFR protein products are about 80% free of other proteins which may be present due to the production technique used in the manufacture of the GDNFR protein product. More preferably, the GDNFR protein products are about 90% free of other proteins, particularly preferably, about 95% free of other proteins,

and most preferably about >98% free of other proteins. In addition, the present invention furnishes the unique advantage of providing polynucleotide sequences for the manufacture of homogeneous GDNFR proteins.

A variety of GDNFR variants are contemplated, including addition, deletion and substitution variants. For example, a series of deletion variants may be made by removing one or more amino acid residues from the amino and/or carboxy termini of the GDNFR protein. Using rules for the prediction of signal peptide cleavage as described by von Heijne (von Heijne, Nucleic Acids Research, 14, 4683-4690, 1986), the first amino acid residue of the GDNFR-α protein which might be involved in GDNF binding is Ser^{18}, as depicted in the full length amino acid sequence of human GDNFR-α in Figure 2 (SEQ ID NO:2). Amino acid residues Met^{1} through Ser^{18} are in the amino-terminal hydrophobic region that is likely to be part of a signal peptide sequence, and therefore, not be included in the mature form of the receptor protein. Similarly, the last amino acid residue of the GDNFR-α protein which is likely to be necessary for GDNF binding is Ser^{446}. Amino acid residues Leu^{447} through Ser^{465} are in the carboxy-terminal hydrophobic region that is involved in the GPI linkage of the protein to the cell surface. Thus, it is contemplated that any or all of the residues from Met^{1} through Ser^{18} and/or Leu^{447} through Ser^{465} (as depicted in Figure 2 (SEQ ID NO:2) may be removed from the protein without affecting GDNF binding to the GDNFR-α protein, thereby leaving a "core" sequence of Ala^{19} through Pro^{446}. Using known analysis techniques, it is further contemplated that N-terminal truncations may include the removal of one or more amino acid residues up to and including Gly^{24}. Thus, GDNFR-α truncation analogs also may include the deletion of one or more amino acid residues from either or both termini such that an amino acid sequence of Asp^{25} through Pro^{446} or Leu^{447} forms the basis for a core molecule. Additional GDNFR-α analogs are contemplated as involving amino acid residues Ser^{18} through Pro^{449} as depicted in the GDNFR-α amino acid sequence of Figure 4 (SEQ ID NO:4) , i.e., deleting one or more amino acid residues from either or both termini involving the hydrophobic regions depicted as amino acid residues Met^{1} through Ser^{18} and/or Pro^{449} through Ser^{468}. Similar analogs may be designed using the amino acid sequences for GRR2 and GRR3, as well as consensus sequences, as depicted in the Figures.

In addition, it is contemplated that one or more amino acid residues may be removed from either or both of the amino and carboxy termini of the GDNFR protein until the first and last cysteine residues in the full length sequence are reached. It is advantageous to retain the cysteine residues for the proper intramolecular binding of the GDNFR protein. As depicted in the full length amino acid sequence of human

GDNFR-α in Figure 2 (SEQ ID NO:2), any or all of amino acid residues from Met^{1} to Asp^{28} may be removed from the amino terminal without removing the first cysteine residue which appears as Cys^{29}. Similarly, any or all of amino acid residues from Gly^{443} to Ser^{465} may be removed from the carboxy terminal without removing the last cysteine residue which appears as Cys^{442}. Other GDNFR-α analogs may be made using amino acid residues Cys^{29} through Cys^{443} as depicted in the GDNFR-α amino acid sequence of Figure 4 (SEQ ID NO:4) , i.e., deleting all or part of the terminal regions depicted as amino acid residues Met^{1} through Asp^{28} and/or Ser^{444} through Ser^{468}. Similar analogs may be designed using the amino acid sequences for GRR2 and GRR3, as well as consensus sequences, as depicted in the Figures.

It will be appreciated by those skilled in the art that, for the same reasons, it is contemplated that these identified amino acid residues may be replaced, rather than deleted, without affecting the function of the GDNFR protein. Alternatively, these identified amino acid residues may be modified by intra-residue insertions or terminal additions without affecting the function of the GDNFR protein. In yet another embodiment, a combination of one or more deletions, substitutions or additions may be made.

The present GDNFR proteins or nucleic acids may be used for methods of treatment, or for methods of manufacturing medicaments for treatment. Such treatment includes conditions characterized by excessive production of GDNF or neurturin, wherein the present GDNFRs, particularly in soluble form, may be used to complex to and therefore inactivate such excessive GDNF or neurturin. This treatment may be accomplished by preparing a soluble receptor (e.g., use of the GDNF or neurturin binding domain) or by preparation of a population of cells containing such GDNFR, and transplanting such cells into the individual in need thereof. The present GDNFR protein products may also be used for treatment of those having defective GDNF and/or neurturin receptors. For example, one may treat an individual having defective GDNFRs by preparation and delivery of a soluble receptor, or by preparation of a population of cells containing such non-defective GDNFR and transplanting such cells into an individual. Or, an individual may have an inadequate number of GDNF or neurturin receptors, and cells containing such receptors may be transplanted in order to increase the number of GDNF or neurturin receptors available to an individual. Such compositions may be used in conjunction with the delivery of GDNF or neurturin. It is also contemplated GDNFR protein products may be used in the treatment of conditions responsive to the activation of the c-ret receptor tyrosine kinase.

In yet another aspect of the present invention, a further advantage to the novel compositions is the use of GDNFR to stabilize GDNF protein or neurturin pharmaceutical compositions. In another aspect of the present invention, a GDNFR may be used to screen compounds for antagonist activity.

Other aspects and advantages of the present invention will be apparent to those skilled in the art. For example, additional uses include new assay systems, transgenic animals and antibody production.

Study Models

The present invention provides for assay systems in which GDNF or neurturin activity or activities similar to GDNF or neurturin activity resulting from exposure to a peptide or non-peptide compound may be detected by measuring an elicited physiological response in a cell or cell line which expresses the GDNFR molecules of the present invention. A physiological response may comprise any of the biological effects of GDNF or neurturin, including but not limited to, dopamine uptake, extension of neurites, increased cell survival or growth, as well as the transcriptional activation of certain nucleic acid sequences (e.g. promoter/enhancer elements as well as structural genes), GDNF-related processing, translation, or phosphorylation, and the induction of secondary processes in response to processes directly or indirectly induced by GDNF, to name but a few.

For example, a model system may be created which may be used to study the effects of excess GDNF activity. In such a system, the response of a cell to GDNF may be increased by engineering an increased number of suitable GDNFRs on the cells of the model system relative to cells which have not been so modified. A system may also be developed to selectively provide an increased number of such GDNFRs on cells which normally express GDNFRs. In order to ensure expression of GDNFR, the GDNFR gene may be placed under the control of a suitable promoter sequence. It may be desirable to put the GDNFR gene under the control of a constitutive and/or tissue specific promoter (including but not limited to the CNS neuron specific enolase, neurofilament, and tyrosine hydroxylase promoter), an inducible promoter (such as the metallothionein promoter), the UV activated promoter in the human immunodeficiency virus long terminal repeat (Valeri et al., 1988, Nature 333:78-81), or the CMV promoter (as contained in pCMX, infra) or a developmentally regulated promoter.

By increasing the number of cellular GDNFRs, the response to endogenous GDNF may be increased. If the model system contains little or no GDNF, GDNF may be added to the system. It may also be desirable to add additional GDNF to the model system in order to evaluate the effects of excess GDNF activity. Over

expressing GDNF (or secreted GDNF) may be one method for studying the effects of elevated levels of GDNF on cells already expressing GDNFR.

GDNFR Therapies

In another aspect, certain conditions may benefit from an increase in GDNF and/or neurturin responsiveness. It may, therefore, be beneficial to increase the number or binding affinity of GDNFRs in patients suffering from conditions responsive to GDNF and/or neurturin therapy. This could be achieved through gene therapy, whereby selective expression of recombinant GDNFR in appropriate cells is achieved, for example, by using GDNFR genes controlled by tissue specific or inducible promoters or by producing localized infection with replication defective viruses carrying a recombinant GDNFR gene.

It is envisioned that conditions which will benefit from GDNFR or combined GDNF or neurturin/GDNFR delivery include, but are not limited to, motor neuron disorders including amyotrophic lateral sclerosis, neurological disorders associated with diabetes, Parkinson's disease, Alzheimer's disease, and Huntington's chorea. Additional indications for the use of GDNFR or combined GDNF or neurturin/GDNFR delivery are described above and further include the treatment of: glaucoma or other diseases and conditions involving retinal ganglion cell degeneration; sensory neuropathy caused by injury to, insults to, or degeneration of, sensory neurons; pathological conditions, such as inherited retinal degenerations and age, disease or injury-related retinopathies, in which photoreceptor degeneration occurs and is responsible for vision loss; and injury or degeneration of inner ear sensory cells, such as hair cells and auditory neurons for preventing and/or treating hearing loss due to variety of causes.

Transgenic Animals

In yet another aspect, a recombinant GDNFR gene may be used to inactivate or "knock out" the endogenous gene (e.g., by homologous recombination) and thereby create a GDNFR deficient cell, tissue, or animal. For example, a recombinant GDNFR-α gene may be engineered to contain an insertional mutation which inactivates GDNFR-α. Such a construct, under the control of a suitable promoter, may be introduced into a cell, such as an embryonic stem cell, by any conventional technique including transfection, transduction, injection, etc. Cells containing the construct may then be selected, for example by G418 resistance. Cells which lack an intact GDNFR-α gene are then identified (e. g., by Southern blotting or Northern blotting or assay of expression). Cells lacking an intact GDNFR-α gene may then be

fused to early embryo cells to generate transgenic animals deficient in GDNFR. A comparison of such an animal with an animal not expressing endogenous GDNF would reveal that either the two phenotypes match completely or that they do not, implying the presence of additional GDNF-like factors or receptors. Such an animal may be used to define specific neuronal populations, or other in vivo processes, normally dependent upon GDNF. Thus, these populations or processes may be expected to be effected if the animal did not express GDNFR-α, and therefore, could not respond to GDNF. Similar constructs may be made and procedures followed for GRR2 and GRR3.

Diagnostic Applications

According to the present invention, GDNFR probes may be used to identify cells and tissues which are responsive to GDNF or neurturin in normal or diseased states. The present invention provides for methods for identifying cells which are responsive to GDNF or neurturin by detecting GDNFR expression in such cells. GDNFR expression may be evidenced by transcription of GDNFR mRNA or production of GDNFR protein. GDNFR expression may be detected using probes which identify GDNFR nucleic acid or protein or by detecting "tag" sequences artificially added to the GDNFR protein.

One variety of probe which may be used to detect GDNFR expression is a nucleic acid probe, which may be used to detect GDNFR-encoding RNA by any method known in the art, including, but not limited to, in situ hybridization, Northern blot analysis, or PCR related techniques. Nucleic acid products of the invention may be labeled with detectable markers (such as radiolabels and non-isotopic labels such as biotin) and employed in hybridization processes to locate the human GDNFR gene position and/or the position of any related gene family in a chromosomal map. They may also be used for identifying human GDNFR gene disorders at the DNA level and used as gene markers for identifying neighboring genes and their disorders. Contemplated herein are kits containing such labeled materials.

Polypeptide products of the invention may be "labeled" by association with a detectable marker substance or label (e.g., a radioactive isotope, a fluorescent or chemiluminescent chemical, an enzyme or other label available to one skilled in the art) to provide reagents useful in detection and quantification of GDNF or neurturin in solid tissue and fluid samples such as blood or urine. Such products may also be used in detecting cells and tissues which are responsive to GDNF or neurturin in normal or diseased states.

Another possible assay for detecting the presence of GDNF or neurturin in a

test sample or screening for the presence of a GDNF-like molecule involves contacting the test sample with a GDNFR protein, suitable for binding GDNF or neurturin, immobilized on a solid phase, thereby producing GDNFR-bound GDNF or neurturin protein. The GDNFR-bound GDNF or neurturin may optionally be contacted with a detection reagent, such as a labeled antibody specific for GDNF or neurturin, thereby forming a detectable product. Such assays may be developed in the form of assay devices for analyzing a test sample. In a basic form, such devices include a solid phase containing or coated with an appropriate GDNFR protein. A method for analyzing a test sample for the presence of GDNF-like protein may involve contacting the sample to an assay reagent comprising GDNFR protein, wherein said GDNFR protein reacts with the GDNF-like protein present in the test sample and produces a detectable reaction product indicative of the presence of GDNF.

The assay reagents provided herein may also be embodied as part of a kit or article of manufacture. Contemplated is an article of manufacture comprising a packaging material and one or more preparations of the presently provided nucleic acid or amino acid sequences. Such packaging material will comprise a label indicating that the preparation is useful for detecting GDNF, neurturin, GDNFR or GDNFR defects in a biological sample. As such, the kit may optionally include materials to carry out such testing, such as reagents useful for performing protein analysis, DNA or RNA hybridization analysis, or PCR analysis on blood, urine, or tissue samples.

<u>Anti-GDNFR Antibody</u>

According to the present invention, GDNFR protein, or fragments or derivatives thereof, may be used as an immunogen to generate anti-GDNFR antibodies. To further improve the likelihood of producing an anti-GDNFR immune response, the amino acid sequence of GDNFR may be analyzed in order to identify portions of the molecule which may be associated with increased immunogenicity. For example, the amino acid sequence may be subjected to computer analysis to identify surface epitopes which present computer-generated plots of hydrophilicity, surface probability, flexibility, antigenic index, amphiphilic helix, amphiphilic sheet, and secondary structure of GDNFR. Alternatively, the amino acid sequences of GDNFR from different species could be compared, and relatively non-homologous regions identified; these non-homologous regions would be more likely to be immunogenic across various species.

Also comprehended are polypeptide fragments duplicating only a part of the continuous amino acid sequence or secondary conformations within GDNFR, which fragments may possess one activity of mammalian GDNFR (e.g., immunological

activity) and not others (e.g., GDNF protein binding activity). Thus, the production of antibodies can include the production of anti-peptide antibodies. The following exemplary peptides were synthesized using GDNFR sequences:

Table 1
GDNFR-α Peptides

SJP-6	H_2N-QSCSTKYRTL-COOH	human GDNFR-α, AA 40-49 (SEQ ID NO:25)
SJP-7	H_2N-CKRGMKKEKN-COOH	human GDNFR-α, AA 89-98 (SEQ ID NO:26)
SJP-8	H_2N-LLEDSPYEPV-COOH	human GDNFR-α, AA 115-124 (SEQ ID NO:27)
SJP-9	H_2N-CSYEERERPN-COOH	rat GDNFR-α, AA 233-242 (SEQ ID NO:28)
SJP-10	H_2N-PAPPVQTTTATTTT-COOH	rat GDNFR-α, AA 356-369 (SEQ ID NO:29)

Peptides SJP-6, 7, and 8 are identical in rat and human GDNFR-α. Peptides SJP-9 and 10 are derived from the rat sequence and are each one amino acid different from human. Both polyclonal and monoclonal antibodies may be made by methods known in the art using these peptides or other portions of GDNFR.

Monoclonal antibodies directed against GDNFR may be prepared by any known technique which provides for the production of antibody molecules by continuous cell lines in culture. For example, the hybridoma technique originally developed by Kohler and Milstein to produce monoclonal antibodies (Nature, 256:495-497, 1975), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today 4:72, 1983), the EBV-hybridoma technique (Cole et al., in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc. pp. 77-96, 1985), and the like, may be used.

Human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies also may be prepared for therapeutic use and may be made by any of numerous techniques known in the art (e.g., Teng et al., Proc. Natl. Acad. Sci. U.S.A., 80:7308-7312, 1983; Kozbor et al., Immunology Today, 4:72-79, 1983; Olsson et al., Meth. Enzymol., 92:3-16, 1982). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., Proc. Natl. Acad. Sci. U.S.A., 81:6851, 1984; Takeda et al., Nature, 314:452, 1985).

Various procedures known in the art also may be used for the production of polyclonal antibodies. For the production of antibody, various host animals including, but not limited to, rabbits, mice, rats, etc., can be immunized by injection with GDNFR protein, or a fragment or derivative thereof. Various adjuvants may be used

to increase the immunological response, depending on the host species selected. Useful adjuvants include, but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

A molecular clone of an antibody to a GDNFR epitope also may be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as high performance liquid chromatography, or a combination thereof, etc. The present invention provides for antibody molecules as well as fragments of such antibody molecules. Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')2 fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

Such selective binding molecules may themselves be alternatives to GDNFR protein, and may be formulated as a pharmaceutical composition.

Recombinant Expression of GDNFR Protein

The present invention provides various polynucleotides encoding GDNFR proteins. The expression product or a derivative thereof is characterized by the ability to bind to GDNF or neurturin so that further interactions with signaling molecules can occur, thereby providing or enhancing GDNF or neurturin activity such as increasing dopamine uptake by dopaminergic cells. The polynucleotides may also be used in cell therapy or gene therapy applications.

According to the present invention, novel GDNFR protein and DNA sequences coding for all or part of such receptors are provided. Novel nucleic acid sequences of the invention are useful in securing expression in procaryotic or eucaryotic host cells of polypeptide products having at least a part of the primary structural conformation and one or more of the biological properties of recombinant human GDNFR. The nucleic acids may be purified and isolated, so that the desired coding region is useful

to produce the present polypeptides. Alternatively, the nucleic acid sequence may be used for diagnostic purposes, as described more fully below. Exemplary DNA sequences of the present invention comprise nucleic acid sequences encoding the GDNFR-α amino acid sequences depicted in Figures 2 and 4 and set forth in SEQ. ID NOs:2 and 4. In addition, DNA sequences disclosed by the present invention include: (a) the GDNFR DNA sequences depicted in the Figures (and complementary strands); (b) a DNA sequence which hybridizes (under hybridization conditions disclosed in the cDNA library screening section below, or equivalent conditions or more stringent conditions) to the DNA sequence in subpart (a) or to fragments thereof; and (c) a DNA sequence which, but for the degeneracy of the genetic code, would hybridize to the DNA sequence in subpart (a). Specifically comprehended in parts (b) and (c) are genomic DNA sequences encoding allelic variant forms of human GDNFR and/or encoding GDNFR from other mammalian species, and manufactured DNA sequences encoding GDNFR, fragments of GDNFR, and analogs of GDNFR which DNA sequences may incorporate codons facilitating transcription and translation of messenger RNA in microbial hosts. Such manufactured sequences may readily be constructed according to the methods known in the art as well as the methods described herein.

Recombinant expression techniques, conducted in accordance with the descriptions set forth herein or other known methods, may be used to produce these polynucleotides and express the various GDNFR proteins. For example, by inserting a nucleic acid sequence which encodes a GDNFR protein into an appropriate vector, one skilled in the art can readily produce large quantities of the desired nucleotide sequence. The sequences can then be used to generate detection probes or amplification primers. Alternatively, a polynucleotide encoding a GDNFR protein can be inserted into an expression vector. By introducing the expression vector into an appropriate host, the desired GDNFR protein may be produced in large amounts.

As further described herein, there are numerous host/vector systems available for the propagation of nucleic acid sequences and/or the production of GDNFR proteins. These include, but are not limited to, plasmid, viral and insertional vectors, and prokaryotic and eukaryotic hosts. One skilled in the art can adapt a host/vector system which is capable of propagating or expressing heterologous DNA to produce or express the sequences of the present invention.

By means of such recombinant techniques, the GDNFR proteins of the present invention are readily produced in commercial quantities with greater purity. Furthermore, it will be appreciated by those skilled in the art that, in view of the present disclosure, the novel nucleic acid sequences include degenerate nucleic acid

sequences encoding the GDNFR proteins specifically set forth in the Figures, sequences encoding variants of GDNFR proteins, and those nucleic acid sequences which hybridize, preferably under stringent hybridization conditions, to complements of these nucleic acid sequences (see, Maniatis et. al., Molecular Cloning (A Laboratory Manual); Cold Spring Harbor Laboratory, pages 387 to 389, 1982.) Exemplary stringent hybridization conditions are hybridization in 4 x SSC at 62-67°C, followed by washing in 0.1 x SSC at 62-67°C for approximately an hour. Alternatively, exemplary stringent hybridization conditions are hybridization in 45-55% formamide, 4 x SSC at 40-45°C. DNA sequences which hybridize to the complementary sequences for GDNFR protein under relaxed hybridization conditions and which encode a GDNFR protein of the present invention are also included herein. Examples of such relaxed stringency hybridization conditions are 4 x SSC at 45-55°C or hybridization with 30-40% formamide at 40-45°C.

Preparation of Polynucleotides Encoding GDNFR

Based upon the disclosure of the present invention, a nucleic acid sequence encoding a full length GDNFR protein or a fragment thereof may readily be prepared or obtained by a variety of means, including, without limitation, chemical synthesis, cDNA or genomic library screening, expression library screening, and/or PCR amplification of cDNA. These methods and others useful for preparing nucleic acid sequences are known in the art and are set forth, for example, by Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), by Ausubel et al., eds (Current Protocols in Molecular Biology, Current Protocols Press, 1994), and by Berger and Kimmel (Methods in Enzymology: Guide to Molecular Cloning Techniques, vol. 152, Academic Press, Inc., San Diego, CA, 1987). Preferred nucleic acid sequences encoding GDNFR are mammalian sequences.

Chemical synthesis of a nucleic acid sequence which encodes a GDNFR protein can also be accomplished using methods known in the art, such as those set forth by Engels et al. (Angew. Chem. Intl. Ed., 28:716-734, 1989). These methods include, inter alia, the phosphotriester, phosphoramidite and H-phosphonate methods of nucleic acid sequence synthesis. A preferred method for such chemical synthesis is polymer-supported synthesis using standard phosphoramidite chemistry. Typically, the DNA encoding the desired polypeptide will be several hundred base pairs (bp) or nucleotides in length. Nucleic acid sequences larger than about 100 nucleotides can be synthesized as several fragments using these methods. The fragments can then be ligated together to form a sequence for the expression of a full length GDNFR protein

or a portion thereof.

Alternatively, a suitable nucleic acid sequence may be obtained by screening an appropriate cDNA library (i.e., a library prepared from one or more tissue source(s) believed to express the protein) or a genomic library (a library prepared from total genomic DNA). The source of the cDNA library is typically a tissue that is believed to express GDNFR in reasonable quantities. Typically, the source of the genomic library is any tissue or tissues from a mammalian species believed to harbor a gene encoding GDNFR. The library can be screened for the presence of the GDNFR cDNA/gene using one or more nucleic acid probes (such as oligonucleotides, cDNA or genomic DNA fragments based upon the presently disclosed sequences) that will hybridize selectively with GDNFR cDNA(s) or gene(s) present in the library. The probes typically used for such library screening usually encode a small region of the GDNFR nucleic acid sequence from the same or a similar species as the species from which the library was prepared. Alternatively, the probes may be degenerate, as discussed herein.

Library screening is typically accomplished by annealing the oligonucleotide probe or cDNA to the clones in the library under conditions of stringency that prevent non-specific binding but permit binding (hybridization) of those clones that have a significant level of homology with the probe or primer. Typical hybridization and washing stringency conditions depend in part on the size (i.e., number of nucleotides in length) of the cDNA or oligonucleotide probe, and whether the probe is degenerate. The probability of obtaining a clone(s) is also considered in designing the hybridization solution (e.g., whether a cDNA or genomic library is being screened; if it is a cDNA library, the probability that the cDNA of interest is present at a high level).

Where DNA fragments (such as cDNAs) are used as probes, typical hybridization conditions include those as set forth in Ausubel et al., eds., supra. After hybridization, the blot containing the library is washed at a suitable stringency, depending on several factors such as probe size, expected homology of probe to clone, type of library being screened, number of clones being screened, and the like. Examples of stringent washing solutions (which are usually low in ionic strength and are used at relatively high temperatures) are as follows. One such stringent wash is 0.015 M NaCl, 0.005 M NaCitrate and 0.1% SDS at 55-65°C. Another such stringent buffer is 1 mM Na_2EDTA, 40 mM $NaHPO_4$, pH 7.2, and 1% SDS at about 40-50°C. One other stringent wash is 0.2 X SSC and 0.1% SDS at about 50-65°C.

There are also exemplary protocols for stringent washing conditions where oligonucleotide probes are used to screen cDNA or genomic libraries. For example, a

first protocol uses 6 X SSC with 0.05 percent sodium pyrophosphate at a temperature of between about 35 and 62°C, depending on the length of the probe. For example, 14 base probes are washed at 35-40°C, 17 base probes at 45-50°C, 20 base probes at 52-57°C, and 23 base probes at 57-63°C. The temperature can be increased 2-3°C where the background non-specific binding appears high. A second protocol uses tetramethylammonium chloride (TMAC) for washing. One such stringent washing solution is 3 M TMAC, 50 mM Tris-HCl, pH 8.0, and 0.2% SDS.

Another suitable method for obtaining a nucleic acid sequence encoding a GDNFR protein is by polymerase chain reaction (PCR). In this method, poly(A)+RNA or total RNA is extracted from a tissue that expresses GDNFR. A cDNA is then prepared from the RNA using the enzyme reverse transcriptase (i.e., RT-PCR). Two primers, typically complementary to two separate regions of the GDNFR cDNA (oligonucleotides), are then added to the cDNA along with a polymerase such as Taq polymerase, and the polymerase amplifies the cDNA region between the two primers.

Where the method of choice for preparing the nucleic acid sequence encoding the desired GDNFR protein requires the use of oligonucleotide primers or probes (e.g., PCR, cDNA or genomic library screening), the oligonucleotide sequences selected as probes or primers should be of adequate length and sufficiently unambiguous so as to minimize the amount of non-specific binding that will occur during library screening or PCR amplification. The actual sequence of the probes or primers is usually based on conserved or highly homologous sequences or regions from the same or a similar gene from another organism, such as the rat nucleic acid sequence involved in the present invention. Optionally, the probes or primers can be fully or partially degenerate, i.e., contain a mixture of probes/primers, all encoding the same amino acid sequence, but using different codons to do so. An alternative to preparing degenerate probes is to place an inosine in some or all of those codon positions that vary by species. The oligonucleotide probes or primers may be prepared by chemical synthesis methods for DNA as described above.

GDNFR proteins based on these nucleic acid sequences encoding GDNFR, as well as mutant or variant sequences thereof, are also contemplated as within the scope of the present invention. Mutant or variant sequences include those sequences containing one or more nucleotide substitutions, deletions, and/or insertions as compared to the wild type sequence and that results in the expression of amino acid sequence variations as compared to the wild type amino acid sequence. In some cases, naturally occurring GDNFR amino acid mutants or variants may exist, due to the existence of natural allelic variation. GDNFR proteins based on such naturally

occurring mutants or variants are also within the scope of the present invention. Preparation of synthetic mutant sequences is also well known in the art, and is described for example in Wells et al. (Gene, 34:315, 1985) and in Sambrook et al., supra.

In some cases, it may be desirable to prepare nucleic acid and/or amino acid variants of naturally occurring GDNFR. Nucleic acid variants (wherein one or more nucleotides are designed to differ from the wild-type or naturally occurring GDNFR) may be produced using site directed mutagenesis or PCR amplification where the primer(s) have the desired point mutations (see Sambrook et al., supra, and Ausubel et al., supra, for descriptions of mutagenesis techniques). Chemical synthesis using methods described by Engels et al., supra, may also be used to prepare such variants. Other methods known to the skilled artisan may be used as well. Preferred nucleic acid variants are those containing nucleotide substitutions accounting for codon preference in the host cell that is to be used to recombinantly produce GDNFR. Other preferred variants are those encoding conservative amino acid changes (e.g., wherein the charge or polarity of the naturally occurring amino acid side chain is not altered substantially by substitution with a different amino acid) as compared to wild type, and/or those designed to either generate a novel glycosylation and/or phosphorylation site(s) on GDNFR, or those designed to delete an existing glycosylation and/or phosphorylation site(s) on GDNFR. Yet other preferred variants are those encoding a GDNFR based upon a GDNFR consensus sequence as depicted in the Figures.

Vectors

The cDNA or genomic DNA encoding the desired GDNFR protein is inserted into a vector for further cloning (amplification of the DNA) or for expression. Suitable vectors are commercially available, or the vector may be specially constructed. Possible vectors include, but are not limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the selected host cell. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322, pUC, or Bluescript® plasmid derivatives (Stratagene, La Jolla CA). The recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, or other known techniques.

For example, the GDNFR-encoding nucleic acid sequence is inserted into a cloning vector which is used to transform, transfect, or infect appropriate host cells so that many copies of the nucleic acid sequence are generated. This can be accomplished by ligating a DNA fragment into a cloning vector which has complementary cohesive termini. If the complementary restriction sites used to fragment the DNA are not

present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. It also may prove advantageous to incorporate restriction endonuclease cleavage sites into the oligonucleotide primers used in polymerase chain reaction to facilitate insertion of the resulting nucleic acid sequence into vectors. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and GDNFR-encoding nucleic acid sequence may be modified by homopolymeric tailing. In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate an isolated GDNFR gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the GDNFR-encoding nucleic acid sequence may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The selection or construction of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the vector, and 3) the host cell (e.g., mammalian, insect, yeast, fungal, plant or bacterial cells) to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and its compatibility with the intended host cell. For DNA expression, the vector components may include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more selection or marker genes, enhancer elements, promoters, a transcription termination sequence, and the like. These components may be obtained from natural sources or synthesized by known procedures. The vectors of the present invention involve a nucleic acid sequence which encodes the GDNFR protein of interest operatively linked to one or more amplification, expression control, regulatory or similar operational elements capable of directing, controlling or otherwise effecting the amplification or expression of the GDNFR-encoding nucleic acid sequence in the selected host cell.

Expression vectors containing GDNFR nucleic acid sequence inserts can be identified by three general approaches: (a) DNA-DNA hybridization; (b) the presence or absence of "marker" gene functions, and (c) the expression of inserted sequences. In the first approach, the presence of a foreign nucleic acid sequence inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to an inserted GDNFR-encoding nucleic acid sequence. In the second approach, the recombinant vector/host system can be

identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of a foreign nucleic acid sequence into the vector. For example, if a GDNFR-encoding nucleic acid sequence is inserted within the marker gene sequence of the vector, recombinants containing the GDNFR insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by detecting the foreign protein product expressed by the recombinant nucleic acid sequence. Such assays can be based on the physical or functional properties of the expressed GDNFR protein product, for example, by binding of the GDNFR-α protein to GDNF or to an antibody which directly recognizes GDNFR-α.

Signal Sequence

The signal sequence may be a component of the vector, or it may be a part of GDNFR DNA that is inserted into the vector. The native GDNFR DNA encodes a signal sequence at the amino terminus of the protein that is cleaved during post-translational processing of the protein to form the mature GDNFR protein. Included within the scope of this invention are GDNFR polynucleotides with the native signal sequence as well as GDNFR polynucleotides wherein the native signal sequence is deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be one that is recognized and processed, i.e., cleaved by a signal peptidase, by the host cell. For prokaryotic host cells that do not recognize and process the native GDNFR signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, or heat-stable enterotoxin II leaders. For yeast secretion, the native GDNFR signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the native signal sequence is satisfactory, although other mammalian signal sequences may be suitable.

Origin of Replication

Expression and cloning vectors generally include a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. In cloning vectors, this sequence is typically one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeasts, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria and various origins (e.g., SV40, polyoma, adenovirus,

VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (for example, the SV40 origin is often used only because it contains the early promoter).

Selection Gene

The expression and cloning vectors may contain a selection gene. This gene encodes a "marker" protein necessary for the survival or growth of the transformed host cells when grown in a selective culture medium. Host cells that were not transformed with the vector will not contain the selection gene, and therefore, they will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients not available from the culture medium.

Other selection genes may be used to amplify the gene which will be expressed. Amplification is the process wherein genes which are in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Examples of suitable selectable markers for mammalian cells include dihydrofolate reductase (DHFR) and thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of the marker present in the vector. Selection pressure is imposed by culturing the transformed cells under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes GDNFR. As a result, increased quantities of GDNFR are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate, a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is used is the Chinese hamster ovary cell line deficient in DHFR activity (see, for example, Urlaub and Chasin, Proc. Natl. Acad. Sci., U.S.A., 77(7): 4216-4220, 1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA present in the expression vector, such as the DNA encoding a GDNFR protein.

Promoter

The expression and cloning vectors of the present invention will typically

contain a promoter that is recognized by the host organism and operably linked to the nucleic acid sequence encoding the GDNFR protein. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as that encoding GDNFR. Promoters are conventionally grouped into one of two classes, inducible promoters and constitutive promoters. Inducible promoters initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, such as the presence or absence of a nutrient or a change in temperature. A large number of promoters, recognized by a variety of potential host cells, are well known. These promoters are operably linked to the DNA encoding GDNFR by removing the promoter from the source DNA by restriction enzyme digestion and inserting the desired promoter sequence into the vector. The native GDNFR promoter sequence may be used to direct amplification and/or expression of GDNFR DNA. A heterologous promoter is preferred, however, if it permits greater transcription and higher yields of the expressed protein as compared to the native promoter, and if it is compatible with the host cell system that has been selected for use.

Promoters suitable for use with prokaryotic hosts include the beta-lactamase and lactose promoter systems; alkaline phosphatase, a tryptophan (trp) promoter system; and hybrid promoters such as the tac promoter. Other known bacterial promoters are also suitable. Their nucleotide sequences have been published, thereby enabling one skilled in the art to ligate them to the desired DNA sequence(s), using linkers or adaptors as needed to supply any required restriction sites.

Suitable promoting sequences for use with yeast hosts are also well known in the art. Yeast enhancers are advantageously used with yeast promoters. Suitable promoters for use with mammalian host cells are well known and include those obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40). Other suitable mammalian promoters include heterologous mammalian promoters, e.g., heat-shock promoters and the actin promoter. A promoter for possible use in the production of GDNFR proteins in CHO cells is SRa (see Takebe et al., Mol. Cell. Biol., 8(1): 466-472, 1988). A suitable expression vector is pDSRa2. The pDSRa2 plasmid constructs containing the appropriate GDNFR cDNA may be prepared substantially in accordance with the process described in the co-owned and copending U. S. Patent Application Serial Number 501,904 filed March 29, 1990 (also see, European Patent Application No. 90305433, Publication No. EP 398 753,

filed May 18, 1990 and WO 90/14363 (1990), the disclosures of which are hereby incorporated by reference.

Additional promoters which may be of interest in controlling GDNFR expression include, but are not limited to: the SV40 early promoter region (Bernoist and Chambon, Nature, 290:304-310, 1981); the CMV promoter; the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., Cell, 22:787-797, 1980); the herpes thymidine kinase promoter (Wagner et al., Proc. Natl. Acad. Sci. U.S.A., 78:144-1445, 1981); the regulatory sequences of the metallothionine gene (Brinster et al., Nature, 296:39-42, 1982); prokaryotic expression vectors such as the beta -lactamase promoter (Villa-Kamaroff, et al., Proc. Natl. Acad. Sci. U.S.A., 75:3727-3731, 1978); or the tac promoter (DeBoer, et al., Proc. Natl. Acad. Sci. U.S.A., 80:21-25, 1983). Also of interest are the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: the elastase I gene control region which is active in pancreatic acinar cells (Swift et al., Cell, 38:639-646, 1984; Ornitz et al., Cold Spring Harbor Symp. Quant. Biol. 50:399-409, 1986; MacDonald, Hepatology, 7:425-515, 1987); the insulin gene control region which is active in pancreatic beta cells (Hanahan, Nature, 315:115-122, 1985); the immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., Cell, 38:647-658, 1984; Adames et al., Nature, 318:533-538, 1985; Alexander et al., Mol. Cell. Biol., 7:1436-1444, 1987); the mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., Cell, 45:485-495, 1986), albumin gene control region which is active in liver (Pinkert et al., Genes and Devel., 1:268-276, 1987); the alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., Mol. Cell. Biol., 5:1639-1648, 1985; Hammer et al., Science, 235:53-58, 1987); the alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., Genes and Devel., 1:161-171, 1987); the beta-globin gene control region which is active in myeloid cells (Mogram et al., Nature, 315:338-340, 1985; Kollias et al., Cell, 46:89-94, 1986); the myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., Cell, 48:703-712, 1987); the myosin light chain-2 gene control region which is active in skeletal muscle (Sani, Nature, 314:283-286, 1985); and the gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., Science, 234:1372-1378, 1986).

<u>Enhancer Element</u>

An enhancer sequence may be inserted into the vector to increase the

transcription of a DNA sequence encoding a GDNFR protein of the present invention by higher eukaryotes. Enhancers are cis-acting elements of DNA, usually about 10-300 bp in length, that act on the promoter to increase its transcription. Enhancers are relatively orientation and position independent. They have been found 5' and 3' to the transcription unit. Several enhancer sequences available from mammalian genes are known (e.g., globin, elastase, albumin, alpha-feto-protein and insulin). Typically, however, an enhancer from a virus will be used. The SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer, and adenovirus enhancers are exemplary enhancing elements for the activation of eukaryotic promoters. While an enhancer may be spliced into the vector at a position 5' or 3' to GDNFR DNA, it is typically located at a site 5' from the promoter.

Transcription Termination

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for terminating transcription and stabilizing the mRNA. Such sequences are commonly available from the 5' and occasionally 3' untranslated regions of eukaryotic DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding GDNFR.

The construction of suitable vectors containing one or more of the above-listed components together with the desired GDNFR-encoding sequence is accomplished by standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the desired order to generate the plasmids required. To confirm that the correct sequences have been constructed, the ligation mixtures may be used to transform E. coli, and successful transformants may be selected by known techniques, such as ampicillin or tetracycline resistance as described above. Plasmids from the transformants may then be prepared, analyzed by restriction endonuclease digestion, and/or sequenced to confirm the presence of the desired construct.

Vectors that provide for the transient expression of DNA encoding GDNFR in mammalian cells may also be used. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of the desired protein encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of proteins encoded by cloned DNAs, as well as

for the rapid screening of such proteins for desired biological or physiological properties. Thus, transient expression systems are particularly useful in identifying variants of the protein.

Selection and Transformation of Host Cells

Host cells (e.g., bacterial, mammalian, insect, yeast, or plant cells) transformed with nucleic acid sequences for use in expressing a recombinant GDNFR protein are also provided by the present invention. The transformed host cell is cultured under appropriate conditions permitting the expression of the nucleic acid sequence. The selection of suitable host cells and methods for transformation, culture, amplification, screening and product production and purification are well known in the art. See for example, Gething and Sambrook, Nature, 293: 620-625 (1981), or alternatively, Kaufman et al., Mol. Cell. Biol., 5 (7): 1750-1759 (1985) or Howley et al., U.S. Pat. No. 4,419,446. Additional exemplary materials and methods are discussed herein. The transformed host cell is cultured in a suitable medium, and the expressed GDNFR protein is then optionally recovered, isolated and purified from the culture medium (or from the cell, if expressed intracellularly) by an appropriate means known to those skilled in the art.

Different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast may be used to produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of the heterologous GDNFR protein. Furthermore, different vector/host expression systems may effect processing reactions such as proteolytic cleavages to different extents.

Suitable host cells for cloning or expressing the vectors disclosed herein are prokaryote, yeast, or higher eukaryote cells. Eukaryotic microbes such as filamentous fungi or yeast may be suitable hosts for the expression of GDNFR proteins. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms, but a number of other genera, species, and strains are well known and commonly available.

Host cells to be used for the expression of glycosylated GDNFR protein are also derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture

might be used, whether such culture involves vertebrate or invertebrate cells, including plant and insect cells. The propagation of vertebrate cells in culture (tissue culture) is a well known procedure. Examples of useful mammalian host cell lines include, but are not limited to, monkey kidney CV1 line transformed by SV40 (COS7), human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture), baby hamster kidney cells, and Chinese hamster ovary cells. Other suitable mammalian cell lines include but are not limited to, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Suitable host cells also include prokaryotic cells. Prokaryotic host cells include, but are not limited to, bacterial cells, such as Gram-negative or Gram-positive organisms, for example, E. coli, Bacilli such as B. subtilis, Pseudomonas species such as P. aeruginosa, Salmonella typhimurium, or Serratia marcescans. For example, the various strains of E. coli (e.g., HB101, DH5a, DH10, and MC1061) are well-known as host cells in the field of biotechnology. Various strains of Streptomyces spp. and the like may also be employed. Presently preferred host cells for producing GDNFR proteins are bacterial cells (e.g., Escherichia coli) and mammalian cells (such as Chinese hamster ovary cells, COS cells, etc.)

The host cells are transfected and preferably transformed with the above-described expression or cloning vectors and cultured in a conventional nutrient medium. The medium may be modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Transfection and transformation are performed using standard techniques which are well known to those skilled in the art and which are selected as appropriate to the host cell involved. For example, for mammalian cells without cell walls, the calcium phosphate precipitation method may be used. Electroporation, micro injection and other known techniques may also be used.

Culturing the Host Cells

Transformed cells used to produce GDNFR proteins of the present invention are cultured in suitable media. The media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as gentamicin), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or other energy source. Other supplements may also be included, at appropriate concentrations, as will be appreciated by those skilled in the art. Suitable culture conditions, such as

temperature, pH, and the like, are also well known to those skilled in the art for use with the selected host cells.

Once the GDNFR protein is produced, it may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. For example, GDNFR-α protein may be isolated by binding to an affinity column comprising GDNF or anti-GDNFR-α antibody bound to a stationary support. Similarly, GRR2 protein may be isolated by binding to an affinity column comprising neurturin or anti-GRR2 antibody bound to a stationary support.

Homologous Recombination

It is further envisioned that GDNFR proteins may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding GDNFR. For example, homologous recombination methods may be used to modify a cell that contains a normally transcriptionally silent GDNFR gene or under expressed gene and thereby produce a cell which expresses GDNFR. Homologous recombination is a technique originally developed for targeting genes to induce or correct mutations in transcriptionally active genes (Kucherlapati, Prog. in Nucl. Acid Res. and Mol. Biol., 36:301, 1989). The basic technique was developed as a method for introducing specific mutations into specific regions of the mammalian genome (Thomas et al., Cell, 44:419-428, 1986; Thomas and Capecchi, Cell, 51:503-512, 1987; Doetschman et al., Proc. Natl. Acad. Sci., 85:8583-8587, 1988) or to correct specific mutations within defective genes (Doetschman et al., Nature, 330:576-578, 1987). Exemplary homologous recombination techniques are described in U.S. 5,272,071 (EP 91 90 3051, EP Publication No. 505 500; PCT/US90/07642, International Publication No. WO 91/09955) the disclosure of which is hereby incorporated by reference.

Through homologous recombination, the DNA sequence to be inserted into the genome can be directed to a specific region of the gene of interest by attaching it to targeting DNA. The targeting DNA is DNA that is complementary (homologous) to a region of the genomic DNA. Small pieces of targeting DNA that are complementary to a specific region of the genome are put in contact with the parental strand during the DNA replication process. It is a general property of DNA that has been inserted into a cell to hybridize, and therefore, recombine with other pieces of endogenous DNA through shared homologous regions. If this complementary strand is attached to an

oligonucleotide that contains a mutation or a different sequence of DNA, it too is incorporated into the newly synthesized strand as a result of the recombination. As a result of the proofreading function, it is possible for the new sequence of DNA to serve as the template. Thus, the transferred DNA is incorporated into the genome.

If the sequence of a particular gene is known, such as the nucleic acid sequence, the pre-pro sequence or expression control sequence of GDNFR presented herein, a piece of DNA that is complementary to a selected region of the gene can be synthesized or otherwise obtained, such as by appropriate restriction of the native DNA at specific recognition sites bounding the region of interest. This piece serves as a targeting sequence upon insertion into the cell and will hybridize to its homologous region within the genome. If this hybridization occurs during DNA replication, this piece of DNA, and any additional sequence attached thereto, will act as an Okazaki fragment and will be backstitched into the newly synthesized daughter strand of DNA.

Attached to these pieces of targeting DNA are regions of DNA which may interact with the expression of a GDNFR protein. For example, a promoter/enhancer element, a suppresser, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired GDNFR protein. The control element does not encode GDNFR, but instead controls a portion of the DNA present in the host cell genome. Thus, the expression of GDNFR proteins may be achieved not by transfection of DNA that encodes the GDNFR gene itself, but rather by the use of targeting DNA (containing regions of homology with the endogenous gene of interest) coupled with DNA regulatory segments that provide the endogenous gene sequence with recognizable signals for transcription of a GDNFR protein.

A. GDNFR variants

As discussed above, the terms "GDNFR analogs" as used herein include polypeptides in which amino acids have been deleted from ("deletion variants"), inserted into ("addition variants"), or substituted for ("substitution variants") residues within the amino acid sequence of naturally-occurring GDNFR polypeptides including those depicted in the Figures. Such variants are prepared by introducing appropriate nucleotide changes into the DNA encoding the polypeptide or by in vitro chemical synthesis of the desired polypeptide. It will be appreciated by those skilled in the art that many combinations of deletions, insertions, and substitutions can be made to an amino acid sequence such as mature human GDNFR provided that the final molecule possesses GDNFR activity.

Based upon the present description of particular GDNFR-α, GRR2 and GRR3

amino acid sequences from multiple species, as well as the consensus sequences derived therefrom, one can readily design and manufacture a variety of nucleic acid sequences suitable for use in the recombinant (e.g., microbial) expression of polypeptides having primary conformations which differ from those depicted in the Figures in terms of the identity or location of one or more residues. Mutagenesis techniques for the replacement, insertion or deletion of one or more selected amino acid residues encoded by the nucleic acid sequences depicted in Figures 2 and 4 are well known to one skilled in the art (e.g., U.S. Pat. No. 4,518,584, the disclosure of which is hereby incorporated by reference.) There are two principal variables in the construction of substitution variants: the location of the mutation site and the nature of the mutation. In designing GDNFR substitution variants, the selection of the mutation site and nature of the mutation will depend on the GDNFR characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid modifications and then with more radical selections depending upon the results achieved, (2) deleting the target amino acid residue, or (3) inserting amino acid residues adjacent to the located site. Conservative changes in from 1 to 30 contiguous amino acids are preferred. N-terminal and C-terminal deletion GDNFR protein variants may also be generated by proteolytic enzymes.

For GDNFR deletion variants, deletions generally range from about 1 to 30 contiguous residues, more usually from about 1 to 10 contiguous residues, and typically from about 1 to 5 contiguous residues. N-terminal, C-terminal and internal intrasequence deletions are contemplated. Deletions may be introduced into regions of the molecule which have low homology with non-human GDNFR to modify the activity of GDNFR. Deletions in areas of substantial homology with non-human GDNFR sequences will be more likely to significantly modify GDNFR biological activity. The number of consecutive deletions typically will be selected so as to preserve the tertiary structure of the GDNFR protein product in the affected domain, e.g., cysteine crosslinking. Non-limiting examples of deletion variants include truncated GDNFR protein products lacking N-terminal or C-terminal amino acid residues. For example, one may prepare a soluble receptor by elimination of the peptide region involved in a glycosyl-phosphatidylinositol (GPI) anchorage of GDNFR receptor to the cytoplasmic membrane.

For GDNFR addition variants, amino acid sequence additions typically include N-and/or C-terminal fusions or terminal additions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as internal or medial additions of single or multiple amino acid residues. Polypeptides of the invention may

also include an initial methionine amino acid residue (at position -1 with respect to the first amino acid residue of the desired polypeptide). Internal additions may range generally from about 1 to 10 contiguous residues, more typically from about 1 to 5 residues, and usually from about 1 to 3 amino acid residues. Examples of N-terminal addition variants include GDNFR with the inclusion of a heterologous N-terminal signal sequence to the N-terminus of GDNFR to facilitate the secretion of mature GDNFR from recombinant host cells and thereby facilitate harvesting or bioavailability. Such signal sequences generally will be obtained from, and thus be homologous to, the intended host cell species. Additions may also include amino acid sequences derived from the sequence of other neurotrophic factors. For example, it is contemplated that a fusion protein of GDNF and GDNFR-α, or neurturin and GRR2, may be produced, with or without a linking sequence, thereby forming a single molecule therapeutic entity.

GDNFR substitution variants have one or more amino acid residues of the GDNFR amino acid sequence removed and a different residue(s) inserted in its place. Such substitution variants include allelic variants, which are characterized by naturally-occurring nucleotide sequence changes in the species population that may or may not result in an amino acid change. As with the other variant forms, substitution variants may involve the replacement of single or contiguous amino acid residues at one or more different locations.

Specific mutations of the GDNFR amino acid sequence may involve modifications to a glycosylation site (e.g., serine, threonine, or asparagine). The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at any asparagine-linked glycosylation recognition site or at any site of the molecule that is modified by addition of an O-linked carbohydrate. An asparagine-linked glycosylation recognition site comprises a tripeptide sequence which is specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either Asn-Xaa-Thr or Asn-Xaa-Ser, where Xaa can be any amino acid other than Pro. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) result in non-glycosylation at the modified tripeptide sequence. Thus, the expression of appropriate altered nucleotide sequences produces variants which are not glycosylated at that site. Alternatively, the GDNFR amino acid sequence may be modified to add glycosylation sites.

One method for identifying GDNFR amino acid residues or regions for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (Science, 244: 1081-1085, 1989). In this method, an amino acid residue or

group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions may then be refined by introducing additional or alternate residues at the sites of substitution. Thus, the target site for introducing an amino acid sequence variation is determined, alanine scanning or random mutagenesis is conducted on the corresponding target codon or region of the DNA sequence, and the expressed GDNFR variants are screened for the optimal combination of desired activity and degree of activity.

The sites of greatest interest for substitutional mutagenesis include sites where the amino acids found in GDNFR proteins from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity. Other sites of interest are those in which particular residues of GDNFR-like proteins, obtained from various species, are identical. Such positions are generally important for the biological activity of a protein. Initially, these sites are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 2 under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more substantial changes (exemplary substitutions) may be introduced, and/or other additions or deletions may be made, and the resulting products are screened for activity.

TABLE 2

Amino Acid Substitutions

Original Residue	Preferred Substitutions	Exemplary Substitutions
Ala (A)	Val	Val; Leu; Ile
Arg (R)	Lys	Lys; Gln; Asn
Asn (N)	Gln	Gln; His; Lys; Arg
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Arg	Asn; Gln; Lys; Arg

Ile (I)	Leu	Leu; Val; Met; Ala; Phe; norleucine
Leu (L)	Ile	norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg	Arg; Gln; Asn
Met (M)	Leu	Leu; Phe; Ile
Phe (F)	Leu	Leu; Val; Ile; Ala
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Phe	Trp; Phe; Thr; Ser
Val (V)	Leu	Ile; Leu; Met; Phe; Ala; norleucine

Conservative modifications to the amino acid sequence (and the corresponding modifications to the encoding nucleic acid sequences) are expected to produce GDNFR protein products having functional and chemical characteristics similar to those of naturally occurring GDNFR. In contrast, substantial modifications in the functional and/or chemical characteristics of GDNFR protein products may be accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues may be divided into groups based on common side chain properties:

1) hydrophobic: norleucine, Met, Ala, Val, Leu, Ile;
2) neutral hydrophilic: Cys, Ser, Thr;
3) acidic: Asp, Glu;
4) basic: Asn, Gln, His, Lys, Arg;
5) residues that influence chain orientation: Gly, Pro; and
6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class. Such substituted residues may be introduced into regions of the human GDNFR protein that are homologous with non-human GDNFR proteins, or into the non-homologous regions of the molecule.

Thus, GDNFR proteins include those biologically active molecules containing all or part of the amino acid sequences as depicted in the Figures, as well as consensus

and modified sequences in which biologically equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. It is also contemplated that the GDNFR proteins, analogs, or fragments or derivatives thereof may be differentially modified during or after translation, e.g., by phosphorylation, glycosylation, crosslinking, acylation, proteolytic cleavage, linkage to an antibody molecule, membrane molecule or other ligand.

B. GDNFR Derivatives

Chemically modified derivatives of GDNFR or GDNFR analogs may be prepared by one of skill in the art based upon the present disclosure. The chemical moieties most suitable for derivatization include water soluble polymers. A water soluble polymer is desirable because the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Preferably, the polymer will be pharmaceutically acceptable for the preparation of a therapeutic product or composition. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer/protein conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. The effectiveness of the derivatization may be ascertained by administering the derivative, in the desired form (e.g., by osmotic pump, or, more preferably, by injection or infusion, or, further formulated for oral, pulmonary or other delivery routes), and determining its effectiveness.

Suitable water soluble polymers include, but are not limited to, polyethylene glycol, copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-

polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 2 kDa and about 100 kDa for ease in handling and manufacturing (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight). Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired; the effects, if any, on biological activity; the ease in handling; the degree or lack of antigenicity and other known effects of polyethylene glycol on a therapeutic protein or variant).

The number of polymer molecules so attached may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra- or some combination of derivatization, with the same or different chemical moieties (e.g., polymers, such as different weights of polyethylene glycols). The proportion of polymer molecules to protein (or peptide) molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted protein or polymer) will be determined by factors such as the desired degree of derivatization (e.g., mono, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art. See for example, EP 0 401 384, the disclosure of which is hereby incorporated by reference (coupling PEG to G-CSF), see also Malik et al., Exp. Hematol., 20: 1028-1035, 1992 (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residue. Those having a free carboxyl group may include aspartic acid residues, glutamic acid residues, and the C-terminal amino acid residue. Sulfhydrl groups may also be used as a reactive group for attaching the polyethylene glycol molecule(s). For therapeutic purposes, attachment at an amino group, such as attachment at the N-terminus or lysine group is preferred. Attachment at residues

important for receptor binding should be avoided if receptor binding is desired.

One may specifically desire an N-terminal chemically modified protein. Using polyethylene glycol as an illustration of the present compositions, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective N-terminal chemical modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved. For example, one may selectively N-terminally pegylate the protein by performing the reaction at a pH which allows one to take advantage of the pKa differences between the e-amino group of the lysine residues and that of the a-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. Using reductive alkylation, the water soluble polymer may be of the type described above, and should have a single reactive aldehyde for coupling to the protein. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may be used.

The present invention contemplates use of derivatives which are prokaryote-expressed GDNFR proteins, or variants thereof, linked to at least one polyethylene glycol molecule, as well as use of GDNFR proteins, or variants thereof, attached to one or more polyethylene glycol molecules via an acyl or alkyl linkage.

Pegylation may be carried out by any of the pegylation reactions known in the art. See, for example: Focus on Growth Factors, 3 (2): 4-10, 1992; EP 0 154 316, the disclosure of which is hereby incorporated by reference; EP 0 401 384; and the other publications cited herein that relate to pegylation. The pegylation may be carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer).

Pegylation by acylation generally involves reacting an active ester derivative of polyethylene glycol (PEG) with the GDNFR protein or variant. Any known or

subsequently discovered reactive PEG molecule may be used to carry out the pegylation of GDNFR protein or variant. A preferred activated PEG ester is PEG esterified to N-hydroxysuccinimide (NHS). As used herein, "acylation" is contemplated to include without limitation the following types of linkages between the therapeutic protein and a water soluble polymer such as PEG: amide, carbamate, urethane, and the like. See Bioconjugate Chem., 5: 133-140, 1994. Reaction conditions may be selected from any of those known in the pegylation art or those subsequently developed, but should avoid conditions such as temperature, solvent, and pH that would inactivate the GDNFR or variant to be modified.

Pegylation by acylation will generally result in a poly-pegylated GDNFR protein or variant. Preferably, the connecting linkage will be an amide. Also preferably, the resulting product will be substantially only (e.g., > 95%) mono, di- or tri-pegylated. However, some species with higher degrees of pegylation may be formed in amounts depending on the specific reaction conditions used. If desired, more purified pegylated species may be separated from the mixture, particularly unreacted species, by standard purification techniques, including, among others, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel filtration chromatography and electrophoresis.

Pegylation by alkylation generally involves reacting a terminal aldehyde derivative of PEG with the GDNFR protein or variant in the presence of a reducing agent. Pegylation by alkylation can also result in poly-pegylated GDNFR protein or variant. In addition, one can manipulate the reaction conditions to favor pegylation substantially only at the a-amino group of the N-terminus of the GDNFR protein or variant (i.e., a mono-pegylated protein). In either case of monopegylation or polypegylation, the PEG groups are preferably attached to the protein via a $-CH_2-NH-$ group. With particular reference to the $-CH_2-$ group, this type of linkage is referred to herein as an "alkyl" linkage.

Derivatization via reductive alkylation to produce a monopegylated product exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization. The reaction is performed at a pH which allows one to take advantage of the pKa differences between the e-amino groups of the lysine residues and that of the a-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer that contains a reactive group such as an aldehyde, to a protein is controlled: the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. In one important aspect, the present invention

contemplates use of a substantially homogeneous preparation of monopolymer/GDNFR protein (or variant) conjugate molecules (meaning GDNFR protein or variant to which a polymer molecule has been attached substantially only (i.e., > 95%) in a single location). More specifically, if polyethylene glycol is used, the present invention also encompasses use of pegylated GDNFR protein or variant lacking possibly antigenic linking groups, and having the polyethylene glycol molecule directly coupled to the GDNFR protein or variant.

Thus, GDNFR protein products according to the present invention include pegylated GDNFR protein or variants, wherein the PEG group(s) is (are) attached via acyl or alkyl groups. As discussed above, such products may be mono-pegylated or poly-pegylated (e.g., containing 2-6, and preferably 2-5, PEG groups). The PEG groups are generally attached to the protein at the a- or e-amino groups of amino acids, but it is also contemplated that the PEG groups could be attached to any amino group attached to the protein, which is sufficiently reactive to become attached to a PEG group under suitable reaction conditions.

The polymer molecules used in both the acylation and alkylation approaches may be selected from among water soluble polymers as described above. The polymer selected should be modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, preferably, so that the degree of polymerization may be controlled as provided for in the present methods. An exemplary reactive PEG aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono C1-C10 alkoxy or aryloxy derivatives thereof (see, U.S. Patent 5,252,714). The polymer may be branched or unbranched. For the acylation reactions, the polymer(s) selected should have a single reactive ester group. For the present reductive alkylation, the polymer(s) selected should have a single reactive aldehyde group. Generally, the water soluble polymer will not be selected from naturally-occurring glycosyl residues since these are usually made more conveniently by mammalian recombinant expression systems. The polymer may be of any molecular weight, and may be branched or unbranched.

An exemplary water-soluble polymer for use herein is polyethylene glycol. As used herein, polyethylene glycol is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono-(C1-C10) alkoxy- or aryloxy-polyethylene glycol.

In general, chemical derivatization may be performed under any suitable condition used to react a biologically active substance with an activated polymer molecule. Methods for preparing a pegylated GDNFR protein product will generally comprise the steps of (a) reacting a GDNFR protein product with polyethylene glycol

(such as a reactive ester or aldehyde derivative of PEG) under conditions whereby the protein becomes attached to one or more PEG groups, and (b) obtaining the reaction product(s). In general, the optimal reaction conditions for the acylation reactions will be determined case-by-case based on known parameters and the desired result. For example, the larger the ratio of PEG:protein, the greater the percentage of poly-pegylated product.

Reductive alkylation to produce a substantially homogeneous population of mono-polymer/GDNFR protein product will generally comprise the steps of: (a) reacting a GDNFR protein or variant with a reactive PEG molecule under reductive alkylation conditions, at a pH suitable to permit selective modification of the a-amino group at the amino terminus of said GDNFR protein or variant; and (b) obtaining the reaction product(s).

For a substantially homogeneous population of mono-polymer/GDNFR protein product, the reductive alkylation reaction conditions are those which permit the selective attachment of the water soluble polymer moiety to the N-terminus of GDNFR protein or variant. Such reaction conditions generally provide for pKa differences between the lysine amino groups and the a-amino group at the N-terminus (the pKa being the pH at which 50% of the amino groups are protonated and 50% are not). The pH also affects the ratio of polymer to protein to be used. In general, if the pH is lower, a larger excess of polymer to protein will be desired (i.e., the less reactive the N-terminal a-amino group, the more polymer needed to achieve optimal conditions). If the pH is higher, the polymer:protein ratio need not be as large (i.e., more reactive groups are available, so fewer polymer molecules are needed). For purposes of the present invention, the pH will generally fall within the range of 3-9, preferably 3-6.

Another important consideration is the molecular weight of the polymer. In general, the higher the molecular weight of the polymer, the fewer polymer molecules may be attached to the protein. Similarly, branching of the polymer should be taken into account when optimizing these parameters. Generally, the higher the molecular weight (or the more branches) the higher the polymer:protein ratio. In general, for the pegylation reactions contemplated herein, the preferred average molecular weight is about 2 kDa to about 100 kDa. The preferred average molecular weight is about 5 kDa to about 50 kDa, particularly preferably about 12 kDa to about 25 kDa. The ratio of water-soluble polymer to GDNF protein or variant will generally range from 1:1 to 100:1, preferably (for polypegylation) 1:1 to 20:1 and (for monopegylation) 1:1 to 5:1.

Using the conditions indicated above, reductive alkylation will provide for selective attachment of the polymer to any GDNFR protein or variant having an a-amino group at the amino terminus, and provide for a substantially homogenous

preparation of monopolymer/GDNFR protein (or variant) conjugate. The term "monopolymer/GDNFR protein (or variant) conjugate" is used here to mean a composition comprised of a single polymer molecule attached to a molecule of GDNFR protein or GDNFR variant protein. The monopolymer/GDNFR protein (or variant) conjugate typically will have a polymer molecule located at the N-terminus, but not on lysine amino side groups. The preparation will generally be greater than 90% monopolymer/GDNFR protein (or variant) conjugate, and more usually greater than 95% monopolymer/GDNFR protein (or variant) conjugate, with the remainder of observable molecules being unreacted (i.e., protein lacking the polymer moiety). It is also envisioned that the GDNFR protein product may involve the preparation of a pegylated molecule involving a fusion protein or linked GDNFR and neurotrophic factor, such as GDNFR-α and GDNF molecules or GRR2 and neurturin molecules.

For the present reductive alkylation, the reducing agent should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation. Suitable reducing agents may be selected from sodium borohydride, sodium cyanoborohydride, dimethylamine borane, trimethylamine borane and pyridine borane. A particularly suitable reducing agent is sodium cyanoborohydride. Other reaction parameters, such as solvent, reaction times, temperatures, etc., and means of purification of products, can be determined case-by-case based on the published information relating to derivatization of proteins with water soluble polymers (see the publications cited herein).

C. <u>GDNFR Protein Product Pharmaceutical Compositions</u>

GDNFR protein product pharmaceutical compositions typically include a therapeutically or prophylactically effective amount of GDNFR protein product in admixture with one or more pharmaceutically and physiologically acceptable formulation materials selected for suitability with the mode of administration. Suitable formulation materials include, but are not limited to, antioxidants, preservatives, coloring, flavoring and diluting agents, emulsifying agents, suspending agents, solvents, fillers, bulking agents, buffers, delivery vehicles, diluents, excipients and/or pharmaceutical adjuvants. For example, a suitable vehicle may be water for injection, physiological saline solution, or artificial cerebrospinal fluid, possibly supplemented with other materials common in compositions for parenteral administration. Neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles. The term "pharmaceutically acceptable carrier" or "physiologically acceptable carrier" as used herein refers to a formulation material(s) suitable for accomplishing or enhancing the delivery of the GDNFR protein product as a pharmaceutical

composition.

The primary solvent in a vehicle may be either aqueous or non-aqueous in nature. In addition, the vehicle may contain other formulation materials for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the vehicle may contain additional formulation materials for modifying or maintaining the rate of release of GDNFR protein product, or for promoting the absorption or penetration of GDNFR protein product across the blood-brain barrier.

Once the therapeutic pharmaceutical composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in a ready to use form or in a form (e.g., lyophilized) requiring reconstitution prior to administration.

The optimal pharmaceutical formulation will be determined by one skilled in the art depending upon the intended route of administration and desired dosage. See for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712, the disclosure of which is hereby incorporated by reference. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present proteins and derivatives.

Effective administration forms, such as (1) slow-release formulations, (2) inhalant mists, or (3) orally active formulations are envisioned. The GDNFR protein product pharmaceutical composition also may be formulated for parenteral administration. Such parenterally administered therapeutic compositions are typically in the form of a pyrogen-free, parenterally acceptable aqueous solution comprising the GDNFR protein product in a pharmaceutically acceptable vehicle. One preferred vehicle is physiological saline. The GDNFR protein product pharmaceutical compositions also may include particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hyaluronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation.

A particularly suitable vehicle for parenteral injection is sterile distilled water in which the GDNFR protein product is formulated as a sterile, isotonic solution, properly preserved. Yet another preparation may involve the formulation of the GDNFR protein product with an agent, such as injectable microspheres or liposomes, that provides for the slow or sustained release of the protein which may then be delivered as a depot injection. Other suitable means for the introduction of GDNFR protein product include implantable drug delivery devices which contain the GDNFR

protein product.

The preparations of the present invention may include other components, for example parenterally acceptable preservatives, tonicity agents, cosolvents, wetting agents, complexing agents, buffering agents, antimicrobials, antioxidants and surfactants, as are well known in the art. For example, suitable tonicity enhancing agents include alkali metal halides (preferably sodium or potassium chloride), mannitol, sorbitol and the like. Suitable preservatives include, but are not limited to, benzalkonium chloride, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid and the like. Hydrogen peroxide may also be used as preservative. Suitable cosolvents are for example glycerin, propylene glycol and polyethylene glycol. Suitable complexing agents are for example caffeine, polyvinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta-cyclodextrin. Suitable surfactants or wetting agents include sorbitan esters, polysorbates such as polysorbate 80, tromethamine, lecithin, cholesterol, tyloxapal and the like. The buffers can be conventional buffers such as borate, citrate, phosphate, bicarbonate, or Tris-HCl.

The formulation components are present in concentration that are acceptable to the site of administration. For example, buffers are used to maintain the composition at physiological pH or at slightly lower pH, typically within a pH range of from about 5 to about 8.

A pharmaceutical composition may be formulated for inhalation. For example, the GDNFR protein product may be formulated as a dry powder for inhalation. GDNFR protein product inhalation solutions may also be formulated in a liquefied propellant for aerosol delivery. In yet another formulation, solutions may be nebulized.

It is also contemplated that certain formulations containing GDNFR protein product are to be administered orally. GDNFR protein product which is administered in this fashion may be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. For example, a capsule may be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional formulation materials may be included to facilitate absorption of GDNFR protein product. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders may also be employed.

Another preparation may involve an effective quantity of GDNFR protein product in a mixture with non-toxic excipients which are suitable for the manufacture of tablets. By dissolving the tablets in sterile water, or other appropriate vehicle,

solutions can be prepared in unit dose form. Suitable excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

Additional GDNFR protein product formulations will be evident to those skilled in the art, including formulations involving GDNFR protein product in combination with GDNF protein product. Techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible microparticles or porous beads and depot injections, are also known to those skilled in the art. See, for example, Supersaxo et al. description of controlled release porous polymeric microparticles for the delivery of pharmaceutical compositions (International Publication No. WO 93/15722; International Application No. PCT/US93/00829) the disclosure of which is hereby incorporated by reference.

D. Administration of GDNFR Protein Product

The GDNFR protein product may be administered parenterally via a variety of routes, including subcutaneous, intramuscular, intravenous, transpulmonary, transdermal, intrathecal and intracerebral delivery. In addition, protein factors that do not readily cross the blood-brain barrier may be given directly intracerebrally or otherwise in association with other elements that will transport them across the barrier. For example, the GDNFR protein product may be administered intracerebroventricularly or into the brain or spinal cord subarachnoid space. GDNFR protein product may also be administered intracerebrally directly into the brain parenchyma. GDNFR protein product may be administered extracerebrally in a form that has been modified chemically or packaged so that it passes the blood-brain barrier, or with one or more agents capable of promoting penetration of GDNFR protein product across the barrier. For example, a conjugate of NGF and monoclonal anti-transferrin receptor antibodies has been shown to be transported to the brain via binding to transferrin receptors.

To achieve the desired level of GDNFR protein product, repeated daily or less frequent injections may be administered, or GDNFR protein product may be infused continuously or periodically from a constant- or programmable-flow implanted pump. Slow-releasing implants containing the neurotrophic factor embedded in a biodegradable polymer matrix can also deliver GDNFR protein product. The frequency of dosing will depend on the pharmacokinetic parameters of the GDNFR protein product as formulated, and the route and site of administration.

Regardless of the manner of administration, the specific dose may be calculated

according to body weight, body surface area or organ size. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them. Appropriate dosages may be ascertained through use of appropriate dose-response data.

The final dosage regimen involved in a method for treating a specific injury or condition will be determined by the attending physician. Generally, an effective amount of the GDNFR protein product will be determined by considering various factors which modify the action of drugs, e.g., the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. See, Remington's Pharmaceutical Sciences, supra, at pages 697-773, herein incorporated by reference. For example, it is contemplated that if GDNFR-α is used to enhance GDNF action, then the GDNFR-α dose is selected to be similar to that required for GDNF therapy; if GDNFR-α is used to antagonize GDNF action, then the GDNFR-α dose would be several times the GDNF dose. Dosing may be one or more times daily, or less frequently, and may be in conjunction with other compositions as described herein. It should be noted that the present invention is not limited to the dosages recited herein.

It is envisioned that the continuous administration or sustained delivery of GDNFR protein products may be advantageous for a given treatment. While continuous administration may be accomplished via a mechanical means, such as with an infusion pump, it is contemplated that other modes of continuous or near continuous administration may be practiced. For example, chemical derivatization or encapsulation may result in sustained release forms of the protein which have the effect of continuous presence in the bloodstream, in predictable amounts, based on a determined dosage regimen. Thus, GDNFR protein products include proteins derivatized or otherwise formulated to effectuate such continuous administration. Sustained release forms of the GDNFR protein products will be formulated to provide the desired daily or weekly effective dosage.

It is further contemplated that the GDNFR protein product may be administered in a combined form with GDNF and/or neurturin. Alternatively, the GDNFR protein product may be administered separately form a neurotrophic factor, either sequentially or simultaneously.

GDNFR protein product of the present invention may also be employed, alone or in combination with other growth factors in the treatment of nerve disease. In addition, other factors or other molecules, including chemical compositions, may be employed together with a GDNFR protein product. For example, in the treatment of

Parkinson's Disease, it is contemplated that GDNFR protein product be used by itself or in conjunction with the administration of Levodopa, wherein the GDNFR would enhance the activity of endogenous GDNF and thereby enhance the neuronal uptake of the increased concentration of dopamine.

As stated above, it is also contemplated that additional neurotrophic or neuron nurturing factors will be useful or necessary to treat some neuronal cell populations or some types of injury or disease. Other factors that may be used in conjunction with GDNFR or a combination of GDNFR and a neurotrophic factor such as GDNF or neurturin include, but are not limited to: mitogens such as insulin, insulin-like growth factors, epidermal growth factor, vasoactive growth factor, pituitary adenylate cyclase activating polypeptide, interferon and somatostatin; neurotrophic factors such as nerve growth factor, brain derived neurotrophic factor, neurotrophin-3, neurotrophin-4/5, neurotrophin-6, insulin-like growth factor, ciliary neurotrophic factor, acidic and basic fibroblast growth factors, fibroblast growth factor-5, transforming growth factor-ß, cocaine-amphetamine regulated transcript (CART); and other growth factors such as epidermal growth factor, leukemia inhibitory factor, interleukins, interferons, and colony stimulating factors; as well as molecules and materials which are the functional equivalents to these factors.

<u>GDNFR Protein Product Cell Therapy and Gene Therapy</u>

GDNFR protein product cell therapy, e.g., intracerebral implantation of cells producing GDNFR protein product, is also contemplated. This embodiment would involve implanting into patients cells capable of synthesizing and secreting a biologically active form of GDNFR protein product. Such GDNFR protein product-producing cells may be cells that are natural producers of GDNFR protein product or may be recombinant cells whose ability to produce GDNFR protein product has been augmented by transformation with a gene encoding the desired GDNFR protein product. Such a modification may be accomplished by means of a vector suitable for delivering the gene as well as promoting its expression and secretion. In order to minimize a potential immunological reaction in patients being administered a GDNFR protein product of a foreign species, it is preferred that the natural cells producing GDNFR protein product be of human origin and produce human GDNFR protein product. Likewise, it is preferred that the recombinant cells producing GDNFR protein product be transformed with an expression vector containing a gene encoding a human GDNFR protein product.

Implanted cells may be encapsulated to avoid infiltration of surrounding tissue. Human or non-human animal cells may be implanted in patients in biocompatible,

semipermeable polymeric enclosures or membranes that allow release of GDNFR protein product, but that prevent destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissue. Alternatively, the patient's own cells, transformed to produce GDNFR protein product ex vivo, could be implanted directly into the patient without such encapsulation.

Techniques for the encapsulation of living cells are familiar to those of ordinary skill in the art, and the preparation of the encapsulated cells and their implantation in patients may be accomplished without undue experimentation. For example, Baetge et al. (International Publication No. WO 95/05452; International Application No. PCT/US94/09299 the disclosure of which is hereby incorporated by reference) describe biocompatible capsules containing genetically engineered cells for the effective delivery of biologically active molecules. In addition, see U.S. Patent Numbers 4,892,538, 5,011,472, and 5,106,627, each of which is specifically incorporated herein by reference. A system for encapsulating living cells is described in PCT Application WO 91/10425 of Aebischer et al., specifically incorporated herein by reference. See also, PCT Application WO 91/10470 of Aebischer et al., Winn et al., Exper. Neurol., 113:322-329, 1991, Aebischer et al., Exper. Neurol., 111:269-275, 1991; Tresco et al., ASAIO, 38:17-23, 1992, each of which is specifically incorporated herein by reference.

In vivo and in vitro gene therapy delivery of GDNFR protein product is also envisioned. In vitro gene therapy may be accomplished by introducing the gene coding for GDNFR protein product into targeted cells via local injection of a nucleic acid construct or other appropriate delivery vectors. (Hefti, J. Neurobiol,. 25:1418-1435, 1994). For example, a nucleic acid sequence encoding a GDNFR protein product may be contained in an adeno-associated virus vector for delivery into the targeted cells (e.g., Johnson, International Publication No. WO 95/34670; International Application No. PCT/US95/07178 the disclosure of which is hereby incorporated by reference). Alternative viral vectors include, but are not limited to, retrovirus, adenovirus, herpes simplex virus and papilloma virus vectors. Physical transfer, either in vivo or ex vivo as appropriate, may also be achieved by liposome-mediated transfer, direct injection (naked DNA), receptor-mediated transfer (ligand-DNA complex), electroporation, calcium phosphate precipitation or microparticle bombardment (gene gun).

It is also contemplated that GDNFR protein product gene therapy or cell therapy can further include the delivery of GDNF protein product. For example, the host cell may be modified to express and release both GDNFR-α protein product and GDNF, or GRR2 and neurturin. Alternatively, the GDNFR-α and GDNF protein

products, or GRR2 and neurturin, may be expressed in and released from separate cells. Such cells may be separately introduced into the patient or the cells may be contained in a single implantable device, such as the encapsulating membrane described above.

It should be noted that the GDNFR protein product formulations described herein may be used for veterinary as well as human applications and that the term "patient" should not be construed in a limiting manner. In the case of veterinary applications, the dosage ranges may be determined as described above.

EXAMPLES

Example 1

Identification of Cells Expressing High Affinity GDNF Binding Sites

Expression cloning involved the selection of a source of mRNA which is likely to contain significant levels of the target transcript. Retina photoreceptor cells were identified as responsive to GDNF at very low concentrations, suggesting the existence of a functional, high affinity receptor. To confirm that rat photoreceptor cells did express a high affinity receptor for GDNF, [^{125}I]GDNF binding and photographic emulsion analysis were carried out.

Rat Retinal Cell Cultures

The neural retinas of 5-day-old C57Bl/6 mouse pups or 3-day-old Sprague-Dawley rat pups (Jackson Laboratories, Bar Harbor, MA) were carefully removed and dissected free of the pigment epithelium, cut into 1 mm^2 fragments and placed into ice-cold phosphate-buffered saline (PBS). The retinas were then transferred into 10 mL of Hank's balanced salt solution (HBSS) containing 120 units papain and 2000 units DNAase and incubated for 20 minutes at 37°C on a rotary platform shaker at about 200 rpm. The cells were then dispersed by trituration through fire-polished Pasteur pipettes, sieved through a 20 μm Nitex nylon mesh and centrifuged for five minutes at 200 x g . The resulting cell pellet was resuspended into HBSS containing 1% ovalbumin and 500 units DNAase, layered on top of a 4 % ovalbumin solution (in HBSS) and centrifuged for 10 minutes at 500 x g. The final pellet was resuspended in complete culture medium (see below), adjusted to about 15,000 cells/mL, and seeded in 90 μl aliquots into tissue culture plates coated with polyornithine and laminin as

previously described (Louis et al., Journal Of Pharmacology And Experimental Therapeutics, 262, 1274-1283, 1992).

The culture medium consisted of a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and F12 medium, and was supplemented with 2.5% heat-inactivated horse serum (Hyclone, Logan, UT), B27 medium supplement (GIBCO, Grand Island, NY), D-glucose (final concentration: 5mg/mL), L-glutamine (final concentration: 2mM), 20 mM HEPES, bovine insulin and human transferrin (final concentrations: 2.5 and 0.1 mg/mL, respectively).

Immunocytochemical identification of photoreceptors

Photoreceptors were identified by immunostaining for arrestin, a rod-specific antigen. Cultures of photoreceptors were fixed for 30 minutes at room temperature with 4% paraformaldehyde in PBS, pH 7.4, followed by three washes in PBS. The fixed cultures were then incubated in Superblock blocking buffer (Pierce, Rockford, IL) , containing 1% Nonidet P-40 to increase the penetration of the antibodies. The anti-arrestin antibodies (polyclonal rabbit antibody against the synthetic peptide sequence of arrestin: Val-Phe-Glu-Glu-Phe-Ala-Arg-Gln-Asn-Leu-Lys-Cys) were then applied at a dilution of between 1:2000 in the same buffer, and the cultures were incubated for one hour at 37°C on a rotary shaker. After three washes with PBS, the cultures were incubated for one hour at 37°C with goat-anti-rabbit IgG (Vectastain kit from Vector Laboratories, Burlingame, CA) at a 1:500 dilution. After three washes with PBS, the secondary antibodies were then labeled with an avidin-biotin-peroxidase complex diluted at 1:500 (45 minutes at 37°C). After three more washes with PBS, the labeled cell cultures were reacted for 5-20 minutes in a solution of 0.1 M Tris-HCl, pH 7.4, containing 0.04% 3',3'-diaminobenzidine-(HCl)4, 0.06 percent $NiCl_2$ and 0.02 percent hydrogen peroxide. Based on arrestin-immunoreactivity, about 90% of the cells in the cultures were rod photoreceptors.

The survival of photoreceptors was determined by examination of arrestin-stained cultures with bright-light optics at 200X magnification. The number of arrestin-positive photoreceptors was counted in one diametrical 1 X 6 mm strip, representing about 20 percent of the total surface area of a 6 mm-well. Viable photoreceptors were characterized as having a regularly-shaped cell body, with a usually short axon-like process. Photoreceptors showing signs of degeneration, such as having irregular, vacuolated perikarya or fragmented neurites, were excluded from the counts (most of the degenerating photoreceptors, however, detached from the culture substratum). Cell numbers were expressed either as cells/6-mm well.

Cultured rat retinal cells enriched for photoreceptors (10,000/6-mm well) were treated with human recombinant GDNF (ten-fold serial dilutions ranging from 10 ng/mL to 1 pg/mL). The cultures were fixed after six days and immunostained for arrestin, a rod photoreceptor-specific antigen. In cultures that were not treated with GDNF, the number of photoreceptors declined steadily over time to reach about 25 percent of the initial number after six days in culture. Treatment of the cultures with GDNF resulted in an about two-fold higher number of viable arrestin-positive photoreceptors after six days in culture. The effect of GDNF was maximal at about 200 pg/mL, with an ED_{50} of about 30 pg/mL. In addition to promoting photoreceptor survival, the addition of the GDNF also stimulated the extension of their axon-like process, thereby demonstrating an effect on the morphological development of the photoreceptors (mean neurite length of photoreceptors in GDNF: 68 μm, compared to 27± 18 μm in control cultures).

In order to confirm that rat retinal cells express high affinity GDNF receptors, [^{125}I]GDNF binding and photographic emulsion analysis were carried out. Post-natal rat photoreceptor cells were seeded on plastic slide flaskettes (Nunc) at a density of 2800 cells/mm2, three to four days before the experiments. The cells were washed once with ice-cold washing buffer (Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.5). For competitive binding, the cells were incubated with various concentrations of [^{125}I]GDNF in binding buffer (DMEM containing 25 mM HEPES, pH 7.5, and 2 mg/mL of bovine serum albumin (BSA)) in the presence or absence of 500 nM unlabeled GDNF at 4°C for four hours. Cells were washed four times with ice-cold washing buffer, lysed in 1 M NaOH and the radioactivity associated with the cells was determined in a gamma counter. A significant amount of [^{125}I]GDNF bound to the photoreceptor cells even at low ligand concentrations (as low as 30 pM), and this binding was inhibited completely by the presence of excess unlabeled GDNF.

For photographic emulsion detection, cells were incubated with 50 pM of [^{125}I]GDNF in binding buffer in the presence or absence of 500 nM unlabeled GDNF at 4°C for four hours. Cells were washed six times with ice-cold washing buffer, fixed with 2.5% glutaraldehyde and dehydrated sequentially with 50% and 70% ethanol, and dipped in NTB-2 photographic emulsion (Eastman Kodak, Rochester NY). After five days of exposure, the slides were developed and examined. The photographic emulsion analysis demonstrated the association of [^{125}I]GDNF to some of the photoreceptor cells, thereby indicating the presence of a receptor for GDNF. This association, however, was efficiently blocked by the addition of unlabeled GDNF.

Example 2

Expression Cloning of a GDNFR-α from Photoreceptor Cells

Rat photoreceptor cells were selected as a possible source of a high affinity receptor for GDNF based upon their cell surface binding of radiolabeled GDNF and their ability to respond to very low concentrations of the ligand, as described in Example 1. In order to identify the receptor, a size-selected cDNA library of approximately 50,000 independent clones was constructed using a mammalian expression vector (a derivative of pSR, Takebe et al., 1988 supra) and mRNA isolated from cultured post-natal rat photoreceptor cells, by the methods described below. The library was divided into pools of approximately 1,500 to 2,000 independent clones and screened using an established expression cloning approach (Gearing et al., EMBO Journal, 8, 3667-3676, 1989). Plasmid DNA representing each pool of the library was prepared and transfected into COS7 cells grown on plastic microscope slide flaskettes (Nunc, Naperville, IL).

The transfected cells were treated with [^{125}I]GDNF, fixed with glutaraldehyde, dehydrated, and dipped in photographic emulsion for autoradiography. Following exposure for five days, the slides were developed and examined for the presence of cells covered by silver grains which indicated the binding of [^{125}I]GDNF to the cell surface as a result of the cell's expression of a receptor for GDNF. EGF receptor transfected cells treated with [^{125}I]EGF were used as a positive control.

One of the 27 pools (F8-11) screened in this manner exhibited 19 positive cells following transfection. Thus, a single cDNA library pool was identified which contained a cDNA clone that expressed GDNFR-α. This pool was divided into 60 smaller subpools of 100 clones/pool which were rescreened by the same procedure described above. Five of these pools were identified as positive and two of the five pools were further subdivided to yield single clones responsible for the GDNF binding activity. Transfection of plasmid DNA from the single clones into COS7 cells resulted in the binding of [^{125}I]GDNF to approximately 15% of the cells. This binding was specifically inhibited by competition with excess unlabeled GDNF.

Construction of Expression cDNA Libraries

Rat retinal cells were harvested from postnatal day 3-7 rats and seeded into culture dishes coated with laminin and polyornithine at a density of approximately

5700 cells/mm^2. After 3-4 days in culture, the population was estimated to contain approximately 80% photoreceptor cells. Total RNA was prepared from this culture by standard methods, and Poly A+ RNA was purified using a polyA-tract kit (Promega, Madison, WI). A cDNA library was constructed from the rat photoreceptor poly A+ RNA using the Gibco Superscript Choice System (Gibco/BRL, Gaithersburg, MD). Two micrograms of poly A+ RNA were mixed with 50 ng of random hexamers, heated to 70^{o}C for 10 minutes and then quick-chilled on ice. First strand synthesis was carried out with 400U Superscript II RT at 37^{o}C for one hour. Second strand synthesis was performed in the same tube after the addition of dNTPs, 10U of E. coli DNA ligase, 40U of E. coli DNA polymerase I, and 2U of E. coli RNase H. After two hours at 16^{o}C, the cDNA ends were blunted by treatment with 10U of T4 polymerase for an additional five minutes at 16^{o}C. Following isopropanol precipitation, EcoRI cloning sites were added to the cDNA by ligation overnight with 10 μg of unphosphorylated EcoRI adapter oligonucleotides.

The EcoRI adapted cDNA was then phosphorylated and applied to a Sephacryl S-500 HR size fractionation column. Following loading, the column was washed with 100 μl aliquots of TEN buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 25 mM NaCl), and 30 μl fractions were collected. Fractions 6 through 8, which contained approximately 34 ng of high molecular weight cDNA, were pooled and precipitated. The recovered EcoRI-adapted cDNA was ligated overnight with 50 ng of EcoRI cut vector pBJ5. Aliquots of the ligation mix containing about 15 ng cDNA each were transformed into competent cells (E. coli strain DH10B; GIBCO/BRL, Gaithersburg, MD) by electroporation. The transformation mixture was titered and then plated on 27 Amp/LB plates at a density of 1500 colonies/plate. Colonies were scraped from each plate and collected into 10 mL of Luria broth (LB) to make 27 pools of 1500 independent clones each. A portion of the cells from each pool was frozen in glycerol and the remainder was used to isolate plasmid DNA using a Qiagen tip-500 kit (Qiagen Inc., Chatsworth, CA).

<u>COS Cell Transfection and Photographic Emulsion Analysis</u>

COS7 cells were seeded (220,000 cells/slide) on plastic slide flaskettes (Nunc) coated with ProNectin (10 μg/mL in phosphate buffered saline (PBS)) one day before transfection. For transfection, 700 μl of Opti MEMI (GIBCO/BRL, Gaithersburg, MD) containing 2 μg cDNA was mixed gently with 35 μl of DEAE Dextran solution (10 mg/mL, Sigma, St. Louis, MO) in an Eppendorf tube. Cells were washed twice with PBS and incubated with the transfection mix for 30 minutes at 37^{o}C in a 5% CO_2 atmosphere. Following incubation, 3 mL of DMEM media containing 10% fetal calf

serum (FCS) and 80 nM Chloroquine (Sigma, St. Louis, MO) were added to each flaskette. Cells were further incubated for 3.5 hours, shocked with 10% dimethylsulfoxide in DMEM at room temperature for two minutes, washed once with PBS, and allowed to grow in DMEM containing 10% FCS. After 48 hours, the transfected COS7 cells were washed once with ice-cold washing buffer (DMEM containing 25 mM HEPES, pH 7.5) and incubated in ice-cold binding buffer (DMEM containing 25 mM HEPES, pH 7.5 and 2 mg/mL BSA) supplemented with 50 pM [^{125}I]GDNF at 4°C for four hours. Cells were washed six times in ice-cold washing buffer, fixed with 2.5% glutaraldehyde at room temperature for five minutes, dehydrated sequentially with 50% and 70% ethanol, and then dipped in NTB-2 photographic emulsion (Eastman Kodak). After 4-5 day exposure at 4°C in dark, the slides were developed and screened by bright-field and dark-field microscopy.

Subdivision of Positive Pools

A single pool was identified which contained a putative GDNF receptor clone. Clones from this pool were plated on 60 plates at a density of 100 colonies/plate. Cells were scraped from each plate, collected in LB, and allowed to grow for 4-5 hours at 37°C. Frozen stocks and DNA preparations were made from each pool, as before, to generate 60 subpools containing 100 independent clones each. Two of these 60 subpools were identified as positive by the method described above, and clones from those pools were plated at low density to allow isolation of single colonies. Single colonies (384) were picked from each of the two subpools and grown for six hours in 200 µl LB in 96-well plates. In order to select single clones expressing GDNFR-α, the four 96-well plates were arrayed into a single large matrix consisting of 16 rows and 24 columns. Cells from the wells in each row and in each column were combined to yield a total of 40 mixtures. These mixtures were grown overnight in 10 mL LB/Amp (100 µg/mL), and DNA was prepared using a Qiagen tip-20 kit. When analyzed for putative GDNF receptor clones, three row mixtures and three column mixtures gave positive signals, suggesting nine potentially positive single clones. DNA from each of the potentially positive single clones was prepared and digested with EcoRI and PstI. DNA from three of the nine single clones exhibited identical restriction patterns while the other six were unrelated, suggesting that the three represented the authentic clones containing GDNFR-α.

Example 3
DNA Sequencing and Sequence Analysis

DNA from positive, single clones was prepared and sequenced using an automated ABI373A DNA sequencer (Perkin/Elmer Applied Biosystems, Santa Clara, CA) and dideoxy-dye-terminators, according to manufacturer's instructions. Comparison of GDNFR-α sequence with all available public databases was performed using the FASTA (Pearson and Lipman, Proceedings Of The National Academy Of Sciences U.S.A., 85, 2444-2448, 1988) program algorithm as described in the University of Wisconsin Genetics Computer Group package (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, Madison, WI).

Sequence Characterization of the Rat GDNFR-α

Plasmid DNA from the clones described in Example 2, above, was prepared and submitted for DNA sequence analysis. Nucleotide sequence analysis of the cloned 2138 bp rat cDNA revealed a single large open reading frame encoding a translation protein of 468 amino acid residues (Figure 3).

The coding sequence is flanked by a 5'-untranslated region of 301 bp and a 3'-untranslated region of 430 bp that does not contain a potential polyadenylation site. The presence of an in-frame stop codon upstream of the first ATG at base pair 302 and its surrounding nucleotide context indicate that this methionine codon is the most likely translation initiator site (Kozak, Nucleic Acids Research. 15, 8125-8148, 1987).

No polyadenylation signal is found in the 430 nucleotides of 3' untranslated sequence in the rat cDNA clone. This is not surprising, since the Northern blot data shows the shortest mRNA transcripts to be approximately 3.6 kb.

The GDNFR-α polypeptide sequence has an N-terminal hydrophobic region of approximately 19 residues (methionine-1 to alanine-19, Figure 3) with the characteristics of a secretory signal peptide (von Heijne, Protein Sequences And Data Analysis. 1, 41-42, 1987; von Heijne, Nucleic Acids Research. 14, 4683-4690, 1986). No internal hydrophobic domain that could serve as a transmembrane domain was found. Instead, a carboxy-terminal hydrophobic region of 21 residues (leucine-448 to serine-468 in Figure 3) is present and may be involved in a glycosyl-phosphatidylinositol (GPI) anchorage of the receptor to the cytoplasmic membrane. Except for the presence of three potential N-linked glycosylation sites, no conserved sequence or structural motifs were found. The protein is extremely rich in cysteine (31 of the 468 amino acid residues) but their spacing is not shared with that of cysteine-

rich domains found in the extracellular portions of known receptors.

The GDNFR-α sequence was compared to sequences in available public databases using FASTA. The search did not reveal significant homology to other published sequences. Once the rat cDNA clone was obtained, it was radiolabeled and used to probe a cDNA library prepared from human brain substantia nigra as described below in Example 5.

Example 4

GDNF Binding to Cells Expressing GDNFR-α

A binding assay was performed in accordance with an assay method previously described by Jing et al.. (Journal Of Cell Biology, 110, 283-294, 1990). The assay involved the binding of [^{125}I]GDNF to rat photoreceptor cells, COS7 cells or 293T cells which had been transfected to express GDNFR-α. Recombinant GDNFR-α expressed on the surface of 293T cells was able to bind GDNF specifically and with an affinity comparable to that observed for GDNF binding sites on rat retinal cells.

Rat photoreceptor cells were prepared as described in Example 1, above, and seeded at a density of 5.7 x 10^5 cells/cm^2 two to three days before the assay in 24-well Costar tissue culture plates pre-coated with polyornithine and laminin. COS7 cells were seeded at a density of 2.5 x 10^4 cells/cm^2 one day before the assay and transfected with 10-20 μg of plasmid DNA using the DEAE-dextran-chloroquine method (Aruffo and Seed, Proceedings Of The National Academy Of Sciences U.S.A., 84, 8573-8577, 1987). Cells from each dish were removed and reseeded into 30 wells of 24-well Costar tissue culture plates 24 hours following the transfection, and allowed to grow for an additional 48 hours. Cells were then left on ice for 5 to 10 minutes, washed once with ice-cold washing buffer and incubated with 0.2 mL of binding buffer containing various concentrations of [^{125}I]GDNF with or without unlabeled GDNF at 4°C for four hours. Cells were washed four times with 0.5 mL ice-cold washing buffer and lysed with 0.5 mL of 1 M NaOH. The lysates were counted in a 1470 Wizard Automatic Gamma Counter.

For some binding experiments, transiently transfected 293T cells were used (see below for 293T cell transfection). Two days following transfection, cells were removed from dishes by 2x versine. Cells were pelleted, washed once with ice-cold binding buffer and resuspended in ice-cold binding buffer at a density of 3 x 10^5 cells/mL. The cell suspension was divided into aliquots containing 1.5 x 10^5

cell/sample. Cells were then pelleted and incubated with various concentrations of [^{125}I]GDNF in the presence or absence of 500 nM of unlabeled GDNF at 4°C for four hours with gentle agitation. Cells were washed four times with ice-cold washing buffer and resuspended in 0.5 mL washing buffer. Two 0.2 mL aliquots of the suspension were counted in a gamma counter to determine the amount of [^{125}I]GDNF associated with the cells.

In all assays, nonspecific binding was determined by using duplicate samples, one of which contained 500 nM of unlabeled GDNF. The level of nonspecific binding varied from 10% to 20% of the specific binding measured in the absence of unlabeled GDNF and was subtracted from the specific binding. The assays demonstrated that cells did not bind GDNF unless the cell had been transfected with the GDNFR-α cDNA clone.

Example 5
Tissue Distribution of GDNFR-α mRNA

The pattern of expression of GDNFR-α mRNA in embryonic mouse, adult mouse, rat, and human tissues was examined by Northern blot analysis. The cloned rat GDNFR-α cDNA was labeled using the Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's procedures. Rat, mouse, and human tissue RNA blots (purchased from Clontech, Palo Alto, CA) were hybridized with the probe and washed using the reagents of the ExpressHyb Kit (Clontech) according to the manufacturer's instructions.

Tissue Northern blots prepared from adult rat, mouse, and human tissues indicated that GDNFR-α mRNA is most highly expressed in liver, brain, and kidney. High mRNA expression was also detected in lung, with lower or non-detectable amounts in spleen, intestine, testis, and skeletal muscle. In blots made from mRNA isolated from mouse embryo, expression was undetectable at embryonic day 7, became apparent at day E11, and was very high by day E17. GDNFR-α mRNA was expressed in tissue isolated from several subregions of adult human brain at relatively equal levels. Expression of GDNFR-α mRNA in human adult brain showed little specificity for any particular region.

In most tissues, transcripts of two distinct sizes were present. In mouse and human tissues, transcripts of 8.5 and 4.4 kb were found, while in rat the transcripts were 8.5 and 3.6 kb. The relative amounts of the larger and smaller transcripts varied with tissue type, the smaller transcript being predominant in liver and kidney and the

larger being more abundant in brain. The binding of GDNF to 293T cells transfected with a GDNFR-α cDNA clone in the pBKRSV vector was examined by Scatchard analysis. Two classes of binding sites were detected, one with a binding affinity in the low picomolar range and another with an affinity of about 500 pM.

Example 6
Recombinant Human GDNFR-α

An adult human substantia nigra cDNA library (5'-stretch plus cDNA library, Clontech, Palo Alto, CA) cloned in bacteriophage gt10 was screened using the rat GDNFR-α cDNA clone of Example 1 as a probe. The probe was labeled with [^{32}P]-dNTPs using a Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Approximately 1.2 x 10^6 gt10 phage from the human substantia nigra cDNA library were plated on 15 cm agarose plates and replicated on duplicate nitrocellulose filters. The filters were then screened by hybridization with the radiolabeled probe. The filters were prehybridized in 200 mL of 6 x SSC, 1 x Denhardts, 0.5% SDS, 50 μg/mL salmon sperm DNA at 55°C for 3.5 hours. Following the addition of 2 x 10^8 cpm of the radiolabeled probe, hybridization was continued for 18 hours. Filters were then washed twice for 30 minutes each in 0.5x SSC, 0.1% SDS at 55°C and exposed to X-ray film overnight with an intensifying screen.

Five positive plaques were isolated whose cDNA inserts represented portions of the human GDNFR-α cDNA. In comparison to the nucleic acid sequence of rat GDNFR-α depicted in Figure 3 (bp 0 through 2140), the five human GDNFR-α clones were found to contain the following sequences:

TABLE 3

Clone 2	1247 through 2330 (SEQ ID NO:21)
Clone 9	1270 through 2330 (SEQ ID NO:23)
Clone 21-A	-235 through 1692 (SEQ ID NO:9)
Clone 21-B	-237 through 1692 (SEQ ID NO:11)
Clone 29	805 through 2971 (SEQ ID NO:15)

An alignment and comparison of the sequences, as depicted in Figure 5, provided a consensus sequence for human GDNFR-α. The translation product predicted by the

human cDNA sequence consists of 465 amino acids and is 93% identical to rat GDNFR-α.

To generate a human cDNA encoding the full length GDNFR-α, portions of clones 21B and 2 were spliced together at an internal BglII site and subcloned into the mammalian expression vector pBKRSV (Stratagene, La Jolla, CA).

Recombinant human GDNFR expression vectors may be prepared for expression in mammalian cells. As indicated above, expression may also be in non-mammalian cells, such as bacterial cells. The nucleic acid sequences disclosed herein may be placed into a commercially available mammalian vector (for example, CEP4, Invitrogen) for expression in mammalian cells, including the commercially available human embryonic kidney cell line, "293". For expression in bacterial cells, one would typically eliminate that portion encoding the leader sequence (e.g., nucleic acids 1-590 of Figure 1). One may add an additional methionyl at the N-terminus for bacterial expression. Additionally, one may substitute the native leader sequence with a different leader sequence, or other sequence for cleavage for ease of expression.

Example 7
Soluble GDNFR Constructs

Soluble human GDNFR protein products were made. The following examples provide four different forms, differing only at the carboxy terminus, indicated by residue numbering as provided in Figure 2. Two are soluble forms truncated at different points just upstream from the hydrophobic tail and downstream from the last cysteine residue. The other two are the same truncations but with the addition of the "FLAG" sequence, an octapeptide to which a commercial antibody is available (Eastman Kodak). The FLAG sequence is H_2N- DYKDDDDK - COOH.

Method

Lambda phage clone #21, containing nearly the entire coding region of human GDNFR-α, was digested with EcoRI to excise the cDNA insert. This fragment was purified and ligated into EcoRI cut pBKRSV vector (Stratagene, La Jolla, CA) to produce the clone 21-B-3/pBKRSV. Primers 1 and 2 as shown below were used in a PCR reaction with the human GDNFR-α clone 21-B-3/pBKRSV as template. PCR conditions were 94°C, five minutes followed by 25 cycles of 94°C, one minute; 55°C, one minute; 72°C, two minutes and a final extension of five minutes at 72°C. This produced a fragment consisting of nucleotides 1265-1868 of the human GDNFR-

α clone plus a termination codon and Hind III restriction site provided by primer 2. This fragment was digested with restriction enzymes Hind III (contained in primer 2) and BglII (position 1304 in human GDNFR-α), and the resulting 572 nucleotide fragment was isolated by gel electrophoresis. This fragment contained the hGDNFR-α- coding region from isoleucine-255 to glycine-443. A similar strategy was used with primers 1 and 3 to produce a fragment with BglII and HindIII ends which coded for isoleucine-255 to proline-446. Primers 4 and 5 were designed to produce fragments coding for the same regions of hGDNFR-α and primers 1 and 3, but with the addition of the Flag peptide coding sequence (IBI/Kodak, New Haven, CN). The Flag peptide sequence consists of eight amino acids (H2N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-COOH) to which antibodies are commercially available. Primers 1 and 4 or 1 and 5 were used in PCR reactions with the same template as before, and digested with HindIII and BglII as before. This procedure produced fragments coding for isoleucine-255 to glycine-443 and isoleucine-255 to proline-446, but with the addition of the Flag peptide at their carboxy termini.

Primers

1) 5'-CTGTTTGAATTTGCAGGACTC-3' (SEQ ID NO:30)

2) 5'-CTCCTCTCTAAGCTTCTAACCACAGCTTGGAGGAGC-3' (SEQ ID NO:31)

3) 5'-CTCCTCTCTAAGCTTCTATGGGCTCAGACCACAGCTT-3' (SEQ ID NO:32)

4) 5'-CTCCTCTCTAAGCTTCTACTTGTCATCGTCGTCCTTGTAGTCACCACAGCTTGGAGGAGC-3' (SEQ ID NO:33)

5) 5'-CTCCTCTCTAAGCTTCTACTTGTCATCGTCGTCCTTGTAGTCTGGCTCAGACCACAGCTT-3' (SEQ ID NO:34)

All four fragments, produced as described above, were transferred back into 21B3/pBKRSV. The 21B3/pBKRSV clone was digested with BglII and HindIII, and treated with calf intestinal alkaline phosphatase (CIAP). The large fragment containing the vector and the human GDNFR-α coding region up to the BglII site was gel purified and extracted from gel. Each of the four BglII/HindIII fragments produced as described above were ligated into this vector resulting in the following constructs in the pBKRSV vector:

TABLE 4

1)	GDNFR-α/gly-443/pBKRSV	hGDNFR-α terminating at glycine 443, followed by stop codon
2)	GDNFR-α/pro-446/pBKRSV	hGDNFR-α terminating at proline 446, followed by stop codon
3)	GDNFR-α/gly-443/Flag/pBKRSV	hGDNFR-α terminating at glycine 443 with C-term Flag tag, followed by stop codon
4)	GDNFR-α/pro-446/Flag/pBKRSV	hGDNFR-α terminating at proline 446 with C-term Flag tag, followed by stop codon

Correct construction of all clones was confirmed by DNA sequencing. Inserts from the pBKRSV clones were transferred to other expression vectors using enzyme sites present in the pBKRSV polylinker sequence as described below. Soluble GDNFRs (e.g., sGDNFR-α/gly and sGDNFR-α/pro) have also been transferred into vectors for transient expression and into pDSR-2 for stable expression in CHO cells.

pDSRα2+PL clones:

The appropriate pBKRSV clone is digested with XbaI and SalI. The insert is ligated to pDSRα2+PL cut with the same enzymes and treated with CIAP. This construction may be used for stable expression of GDNFR in CHO cells.

pCEP4 clones:

The appropriate pBKRSV clone is digested with SpeI and XhoI. The insert is ligated to pCEP4 (Invitrogen, San Diego, CA) digested with NheI (SpeI ends) and XhoI, and treated with CIAP. This construction may be used for transient of expression of GDNFR.

The plasmid construct pDSR–2 is prepared substantially in accordance with the process described in the co-owned and copending U. S. Patent Application Serial Number 07/501,904 filed March 29, 1990 (now abandoned) (also see, European Patent Application No. 90305433, Publication No. EP 398 753, filed May 18, 1990 and WO 90/14363 (1990), the disclosures of which are hereby incorporated by reference. It will be appreciated by those skilled in the art that a variety of nucleic acid sequences encoding GDNFR analogs may be used.

Another construct is pDSRα2, a derivative of the plasmid pCD (Okayama & Berg, Mol. Cell Biol. 3: 280-289, 1983) with three main modifications: (i) the SV40

polyadenylation signal has been replaced with the signal from the α-subunit of bovine follicular stimulating hormone, α-bFSH (Goodwin et al., Nucleic Acids Res. 11: 6873-6882, 1983); (ii) a mouse dihydrofolate reductase minigene (Gasser et al., Proc. Natl. Acad. Sci. 79: 6522-6526, 1982) has been inserted downstream from the expression cassette to allow selection and amplification of the transformants; and (iii) a 267 bp fragment containing the "R-element" and part of the "U5" sequences of the long terminal repeat (LTR) of human T-cell leukemia virus type I (HTLV-I) has been cloned and inserted between the SV40 promoter and the splice signals as described previously (Takebe et al., Mol. Cell Biol. 8: 466-472, 1988).

The expression of GDNFR-α in CHO cells has been verified by the binding of iodinated GDNF to the cell surface. As discussed above, the recombinantly expressed soluble GDNFR-α protein product may be used to potentiate the activity or cell specificity of GDNF. Soluble GDNFR-α attached to a detectable label also may be used in diagnostic applications as discussed above.

Example 8
Chemical Crosslinking of GDNF with GDNFR-α

In order to study its binding properties and molecular characteristics, GDNFR-α was transiently expressed on the surface of 293T cells by transfection of the rat cDNA clone. Transfection of 293T cells was performed using the Calcium Phosphate Transfection System (GIBCO/BRL, Gaithersburg, MD) according to the manufacturers instructions. Two days following transfection, cells were removed by 2x versine treatment, washed once with washing buffer, and resuspended in washing buffer at a density of 2 x 10^6 cells/mL. A duplicate set of cells were incubated with 0.5 u/mL PI-PLC at 37°C for 30 minutes before [^{125}I]GDNF binding. These cells were washed three times with ice-cold binding buffer and then incubated with 1 to 3 nM of [^{125}I]GDNF along with other cells at 4°C for four hours. Cells were washed four times with ice-cold washing buffer, resuspended in washing buffer supplemented with 1 mM of Bis suberate for crosslinking (BS^3 Pierce, Rockford, IL) and incubated at room temperature for 30 minutes. Following three washes with TBS, a duplicate group of samples was treated by 0.5 u/mL of PI-PLC at 37°C for 30 minutes. These cells were pelleted and the supernatants were collected. The cells were then washed with washing buffer and lysed along with all other cells with 2x SDS-PAGE sample buffer. The cell lysates and the collected supernatants were resolved on a 7.5% SDS-PAGE.

The cell suspension was divided into aliquots containing 1.5 x 10^5 cell/sample. Cells were then pelleted and incubated with various concentrations of [^{125}I]GDNF in the presence or absence of 500 nM of unlabeled GDNF at 4°C for four hours with gentle agitation. Cells were washed four times with ice-cold washing buffer and resuspended in 0.5 mL washing buffer. Two 0.2 mL aliquots of the suspension were counted in a gamma counter to determine the amount of [^{125}I]GDNF associated with the cells.

Although mock transfected 293T cells did not exhibit any GDNF binding capacity, GDNFR-α transfected cells bound [^{125}I]GDNF strongly even at picomolar concentrations. This binding was almost completely inhibited by 500 nM of unlabeled GDNF, indicating a specific binding of native GDNF to the expressed receptors.

GDNFR-α expressed by the 293T cells can be released from the cells by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC, Boehringer Mannheim, Indianapolis, IN). The treatment of transfected cells with PI-PLC prior to ligand binding almost entirely eliminated the GDNF binding capacity of the cell. Additionally, treatment of the transfected cells after cross-linking released the majority of the cross-linked products into the media. These results strongly suggest that GDNFR-α is anchored to the cell membrane through a GPI linkage.

Crosslinking data further indicated that the molecular weight of GDNFR-α is approximately 50-65 kD, suggesting that there is a low level of glycosylation. Although the major cross-linked species has a molecular mass consistent with a monomer of the receptor, a minor species with approximately the mass expected for a dimer has been found.

Example 9
GDNF Signaling is Mediated by a Complex of GDNFR-α and the Ret Receptor Protein Tyrosine Kinase

Introduction

Mice carrying targeted null mutations in the GDNF gene exhibit various defects in tissues derived from neural crest cells, in the autonomic nervous system, and in trigeminal and spinal cord motor neurons. The most severe defects are the absence of kidneys and complete loss of enteric neurons in digestive tract. The phenotype of GDNF knockout mice is strikingly similar to that of the c-ret knockout animals (Schuchardt et al. 1994), suggesting a possible linkage between the signal

transduction pathways of GDNF and c-ret.

The proto-oncogene c-ret was identified using probes derived from an oncogene isolated in gene transfer experiments (Takahashi et al., Cell. 42, 581-588, 1985; Takahashi and Cooper, Mol. Cell. Biol., 7, 1378-1385, 1987). Sequence analysis of the c-ret cDNA revealed a large open reading frame encoding a novel receptor protein tyrosine kinase (PTK). The family of receptor PTKs has been grouped into sub-families according to extracellular domain structure and sequence homology within the intracellular kinase domain (van der Geer et al., 1994). The unique extracellular domain structure of Ret places it outside any other known receptor PTK sub-family; it includes a signal peptide, a cadherin-like motif, and a cysteine-rich region (van Heyningen, Nature, 367, 319-320, 1994; Iwamoto et al., 1993). *In situ* hybridization and immunohistochemical analysis showed high level expression of ret mRNA and protein in the developing central and peripheral nervous systems and in the excretory system of the mouse embryo (Pachnis et al., 1993; Tsuzuki et al., Oncogene, 10, 191-198, 1995), suggesting a role of the Ret receptor either in the development or in the function of these tissues. A functional ligand of the Ret receptor has not been identified, thereby limiting a further understanding of the molecular mechanism of Ret signaling.

Mutations in the c-ret gene are associated with inherited predisposition to cancer in familial medullary thyroid carcinoma (FMTC), and multiple endocrine neoplasia type 2A (MEN2A) and 2B (MEN2B). These diseases are probably caused by "gain of function" mutations that constitutively activate the Ret kinase (Donis-Keller et al., Hum. Molec. Genet. 2, 851-856, 1993; Hofstra et al., Nature. 367, 375-376, 1994; Mulligan et al., Nature. 363, 458-460, 1993; Santoro et al., Science. 267, 381-383, 1995). They confer a predisposition to malignancy specifically in tissues derived from the neural crest, where ret is normally expressed in early development. Another ret-associated genetic disorder, Hirschsprung's disease (HSCR), is characterized by the congenital absence of parasympathetic innervation in the lower intestinal tract (Edery et al., Nature. 367, 378-380, 1994; Romeo et al., 1994). The most likely causes of HSCR are nonsense mutations that result in the production of truncated Ret protein lacking a kinase domain or missense mutations that inactivate the Ret kinase. As noted above, targeted disruption of the c-ret proto-oncogene in mice results in renal agenesis or severe dysgenesis and lack of enteric neurons throughout the digestive tract (Schuchardt et al., 1994). This phenotype closely resembles that of GDNF knockout mice. Taken together, these data suggest that both Ret and GDNF are involved in signal transduction pathways critical to the development of the kidney and the enteric nervous system. How Ret and GDNF are

involved, however, was not known.

The isolation and characterization of cDNA for GDNFR-α by expression cloning, as described above, lead to the expression of GDNFR-α in the transformed human embryonic kidney cell line 293T. Transformation resulted in the appearance of both high (K_d of approximately 2 pM) and low (K_d of approximately 200 pM) affinity binding sites. The high affinity binding sites could be composed of homodimers or homo-oligomers of GDNFR-α alone, or of heterodimers or hetero-oligomers of GDNFR-α with other molecules. As discussed above, because GDNFR-α lacks a cytoplasmic domain, it must function through one or more accessory molecules in order to play a role in GDNF signal transduction. In this study we confirm that, in the presence of GDNFR-α, GDNF associates with the Ret protein tyrosine kinase receptor, and quickly induces Ret autophosphorylation.

Results

Neuro-2a Cells Expressing GDNFR-α Bind GDNF with High Affinity

Neuro-2a is a mouse neuroblastoma cell line that endogenously expresses a high level of Ret protein (Ikeda et al., Oncogene. 5, 1291-1296, 1990; Iwamoto et al., Oncogene. 8, 1087-1091, 1993; Takahashi and Cooper, 1987) but does not express detectable levels of GDNFR-α mRNA as judged by Northern blot. In order to determine if Ret could associate with GDNF in the presence of GDNFR-α, a study was performed to examine the binding of [^{125}I]GDNF to Neuro-2a cells engineered to express GDNFR-α. Neuro-2a cells were transfected with a mammalian expression vector containing the rat GDNFR-α cDNA (such as the expression plasmid described above). Three clonal lines, NGR-16, NGR-33, and NGR-38 were tested for their ability to bind [^{125}I]GDNF. The unbound [^{125}I]GDNF was removed at the end of the incubation and the amount of radioactivity associated with the cells was determined as described in Experimental Procedures. All three lines were able to bind [^{125}I]GDNF specifically while parental Neuro-2a cells exhibited little or no [^{125}I]GDNF binding (Figure 6). Binding could be effectively competed by the addition of 500 nM unlabeled GDNF. These results demonstrate that Ret receptor expressed on Neuro-2a cells is unable to bind GDNF in the absence of GDNFR-α and are consistent with the previous observation that GDNFR-α is not expressed at appreciable levels in Neuro-2a cells.

Equilibrium binding of [^{125}I]GDNF to NGR-38 cells was examined over a wide range of ligand concentrations (0.5 pM to 1 nM of [^{125}I]GDNF in the presence or absence of 500 nM of unlabeled GDNF) (see Figure 7A). Following incubation,

unbound [^{125}I]GDNF was removed and the radioactivity associated with the cells was determined as described in Experimental Procedures. Results are depicted in Figure 7: (A) Equilibrium binding of [^{125}I]GDNF to NGR-38 cells (circles) and Neuro-2a cells (squares) in the presence (open circles and open squares) or absence (filled circles and filled squares) of unlabeled GDNF; (B) Scatchard analysis of [^{125}I]GDNF binding to NGR-38 cells. Neuro-2a cells exhibited little binding even at a concentration of 1 nM [^{125}I]GDNF, and this binding was not affected by the addition of excess unlabeled GDNF. Binding to NGR-38 cells was analyzed by Scatchard plot as shown in Figure 7B. Two classes of binding sites were detected, one with $K_d = 1.5 \pm 0.5$ pM and the other with $K_d = 332 \pm 53$ pM. These dissociation constants are very similar to the values obtained for the high and low affinity binding sites in 293T cells transiently expressing GDNFR-α, as described above.

GDNF Associates with Ret in Neuro-2a Cells Expressing GDNFR-α

In order to determine if the Ret receptor PTK could associate with GDNF in cells expressing GDNFR-α, a cross-linking experiment was carried out using NGR-38 and parental Neuro-2a cells. NGR-38 cells were incubated with [^{125}I]GDNF, treated with cross-linking reagent, then lysed either directly in SDS-PAGE sample buffer or in Triton X-100 lysis buffer and further immunoprecipitated with anti-Ret antibody as described in the Experimental Procedures. The immunoprecipitates were analyzed by SDS-PAGE in the absence (NR) or presence (R) of -mercaptoethanol. Lysates were treated with Ret specific antibody, immunoprecipitated, and analyzed by SDS-PAGE under reducing conditions (see Figure 8, bands are marked as follows: ~75 kD, solid triangle; ~150 kD, open triangle; ~185 kD, solid arrow; ~250 kD, asterisk; ~400kD, open arrow). The most prominent cross-linked species were at ~75 kD, and ~185 kD, with less intense bands of ~150 kD and ~250 kD. A very faint band of ~400 kD was also visible (Figure 8, lane 2). When immunoprecipitates were analyzed by non-reducing SDS-PAGE, the ~75 kD, ~150 and ~185 kD bands were present at about the same intensity as in the reducing gel, but the amount of the ~400 kD band increased dramatically (Figure 8, lane 4). Also becoming more prominent was the band at ~250 kD.

Under both reducing and non-reducing conditions, bands of similar molecular weight but of greatly reduced intensity were observed when parental Neuro-2a cells were used instead of NGR-38 (Figure 8, lanes 1 and 3). The ~75 kD and ~150 kD species are likely to represent cross-linked complexes of GDNF and GDNFR-α, since species with identical molecular weights are produced by cross-linking in 293T cells that do not express Ret. Furthermore, since the molecular weight of Ret is 170 kD,

any complex including Ret must be of at least this size.

The fact that these complexes are immunoprecipitated by anti-Ret antibody indicates they are products of an association between Ret and the GDNF/GDNFR-α complex which was disrupted under the conditions of the gel analysis. It is envisioned that the broad band at ~185 kD probably consists of one molecule of Ret (170 kD) cross-linked with one molecule of monomeric recombinant GDNF (15 kD), although some dimeric GDNF may be included. The presence of Ret in this species was confirmed by a separate experiment in which a band of the same molecular weight was observed when unlabeled GDNF was cross-linked to NGR-38 cells and the products examined by Western blot with anti-Ret antibody (data not shown).

The ~400 kD band was not reliably identified, partly due to the difficulty in estimating its molecular weight. The fact that it is prominent only under non-reducing conditions indicates that it is a disulfide-linked dimer of one or more of the species observed under reducing conditions. The most likely explanation is that it represents a dimer of the 185 kD species, although it may be a mixture of high molecular weight complexes consisting of two Ret, one or two GDNFR-α, and one or two GDNF molecules. The exact identity of the ~250 kD band has not yet been determined. One possibility is that it represents cross-linked heterodimers of the ~75 kD (GDNF + GDNFR-α) and ~185 kD (GDNF + Ret) complexes.

GDNF Stimulates Autophosphorylation of Ret in Neuro-2a Cells Expressing GDNFR-α

The ability of the Ret protein tyrosine kinase receptor to associate with GDNF in the presence of GDNFR-α led to the study of GDNF stimulation of the autophosphorylation of Ret. NGR-38 cells were treated with GDNF, lysed, and the lysates immunoprecipitated with anti-Ret antibody. The immunoprecipitates were analyzed by Western blot using an anti-phosphotyrosine antibody as described in the Experimental Procedures. When NGR-38 cells (Figure 9A, lanes 2-4) were treated with purified recombinant GDNF produced in either mammalian (CHO cells; Figure 9A, lanes 4) or E. coli cells (Figure 9A, lanes 1, 3), a strong band was observed at 170 kD, indicating autophosphorylation of tyrosine residues on the mature form of Ret. A much weaker corresponding band was observed in GDNF-treated Neuro-2a cells (Figure 9A, lane 1). No phosphorylation was observed on the alternatively glycosylated 150 kD precursor form of Ret (Figure 9A). The induction of Ret autophosphorylation by GDNF was dosage dependent. The dose response and kinetics of GDNF-induced Ret tyrosine phosphorylation in NGR-38 cells are shown in panels B and C. In all panels, the tyrosine phosphorylated 170 kD Ret bands are

indicated by solid arrows. The amount of Ret protein loaded in each lane as determined by reprobing of the immunoblot with anti-Ret antibody (Santa Cruz, C-19, Cat. #sc-167) is shown on the right side of panel A. The band at ~150 kD represents an alternately glycosylated immature form of Ret that does not autophosphorylate. As shown in Figure 9B, stimulation of Ret autophosphorylation in NGR-38 cells could be detected with 50 pg/mL of GDNF and the response was saturated at 20-50 ng/mL GDNF. The stimulation of Ret autophosphorylation by purified recombinant GDNF in NGR-38 cells over times of 0-20 minutes following treatment is shown in Figure 9C. Increased levels of Ret autophosphorylation could be observed within one minute of GDNF treatment and was maximal at 10 minutes following treatment (Figure 9C).

GDNF and Soluble GDNFR-α Induce Ret Autophosphorylation in Neuro-2A Cells

As discussed above, GDNFR-α is anchored to the cytoplasmic membrane through a GPI linkage and can be released by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC). When NGR-38 cells were incubated with PI-PLC, GDNF-induced receptor autophosphorylation of Ret in these cells was abolished (Figure 10A; PI-PLC treated (lane 1) or untreated (lanes 2 and 3) NGR-38 cells were incubated with (lanes 1 and 3) or without (lane 2) GDNF and analyzed for Ret autophosphorylation by immunoblotting as described in the Experimental Procedures).

Figure 10B depicts parental Neuro-2a cells treated with (lanes 2,4,6,8) or without (lanes 1,3,5,7) GDNF in the presence (lanes 5-8) or absence (lanes 1-4) of PI-PLC/CM obtained from Neuro-2a or NGR-38 cells, as analyzed for Ret autophosphorylation by immunoblotting as described in the Experimental Procedures. NGR-38 cells treated with GDNF were used as a positive control. In both panels A and B, the autophosphorylated 170 kD Ret bands are marked by solid arrows. When conditioned medium containing soluble GDNFR-α released by PI-PLC treatment (PI-PLC/CM) of NGR-38 cells was added to parental Neuro-2a cells along with GDNF, autophosphorylation of the Ret receptor comparable to that obtained with GDNF treatment of NGR-38 cells was observed (Figure 10B, lanes 2 and 8). Only background levels of Ret autophosphorylation were observed when no GDNF was added, or when conditioned media derived from PI-PLC treatment of Neuro-2a cells was tested (Figure 10B, lanes 3-7).

Ret-Fc Fusion Protein Blocks Ret Phosphorylation Induced by GDNF and Soluble GDNFR-α

To confirm that Ret phosphorylation induced by GDNF in the presence of

GDNFR-α is the result of receptor autophosphorylation, a study was performed to determine whether a Ret extracellular domain/Immunoglobulin Fc (Ret-Fc) fusion protein could block Ret activation. Because of the technical difficulty of blocking the large number of GDNF alpha receptors expressed on NGR-38 cells, Ret phosphorylation assays were performed using Neuro-2a as the target cell and culture media removed from NGR-38 cells treated with PI-PLC as a source of GDNFR-α. Cells were treated with mixtures including various combinations of GDNF (50 ng/mL), media containing soluble GDNFR-α (e.g., PI-PLC/CM derived from NGR-38 cells), and different concentrations of Ret-Fc fusion protein either alone or in various combinations as indicated in Figure 11. Neuro-2a cells were treated with GDNF, media containing soluble GDNFR-α, Ret-Fc, or the pre-incubated mixtures. The cells were then lysed, and the lysates were analyzed for c-Ret autophosphorylation by immunoprecipitation using anti-Ret antibody as described in the Experimental Procedures. The immunoprecipitates were analyzed by Western blot using an anti-phosphotyrosine antibody.

The pre-incubated mixture of GDNF and media containing soluble GDNFR-α induced tyrosine phosphorylation of Ret receptors expressed in Neuro-2a at a level comparable to GDNF-treated NGR-38 control cells (Figure 11, lanes 7 and 2). The position of the autophosphorylated 170 kD Ret bands are marked by a solid arrow. When Ret-Fc fusion protein was included in the pre-incubated GDNF/GDNFR-α mixture, Ret phosphorylation was inhibited in a dose dependent manner (Figure 11, lanes 8-10). This indicated that Ret phosphorylation is a result of a GDNF/Ret interaction mediated by GDNFR-α. In untreated Neuro-2a cells or in cells treated with any combination of GDNF or Ret-Fc fusion protein in the absence of GDNFR-α, only background levels of Ret phosphorylation were observed (Figure 11, lanes 3-6).

<u>GDNF Induces Autophosphorylation of c-RET Expressed in Embryonic Motor Neurons</u>

Spinal cord motor neurons are one of the major targets of GDNF action in vivo (Henderson et al., Science. 266, 1062-1064, 1994; Li et al., Proceedings Of The National Academy Of Sciences, U.S.A. 92, 9771-9775, 1995; Oppenheim et al., Nature. 373, 344-346, 1995; Yan et al., Nature. 373, 341-344, 1995; Zurn et al., Neuroreport. 6, 113-118, 1995). To test the ability of GDNF to induce Ret autophosphorylation in these cells, embryonic rat spinal cord motor neurons were treated with (lanes 2 and 4) or without (lanes 1 and 3) 20 ng/mL GDNF followed by lysis of the cells, immunoprecipitation with anti-Ret antibody, and analysis by Western blotting with anti-phosphotyrosine antibody as described in the Experimental

Procedures. In lysates of cells treated with GDNF, a band of tyrosine phosphorylated protein with a molecular mass of ~170 kD was observed (Figure 12, lane 2). No such signal was observed with cells treated with binding buffer alone (Figure 12, lane 1). When the same Western blot filter was stripped and re-probed with anti-Ret antibody (i.e., the amount of c-Ret protein loaded in each lane was determined by reprobing the immunoblot with the anti-Ret antibody), bands with the same molecular mass and similar intensities appeared in both samples (Figure 12, lanes 3 and 4). The phosphotyrosine band in GDNF-treated cells co-migrates with the Ret protein band, indicating GDNF stimulated autophosphorylation of Ret. The autophosphorylated Ret bands (lanes 1 and 2) and the corresponding protein bands (lanes 3 and 4) were marked by a solid arrow.

Discussion

Polypeptide growth factors elicit biological effects through binding to their cognate cell surface receptors. Receptors can be grouped into several classes based on their structure and mechanism of action. These classifications include the protein tyrosine kinases (PTKs), the serine/threonine kinases, and the cytokine receptors. Receptor PTK signaling is initiated by a direct interaction with ligand, which induces receptor dimerization or oligomerization that in turn leads to receptor autophosphorylation. The activated receptor then recruits and phosphorylates intracellular substrates, initiating a cascade of events which culminates in a biological response (Schlessinger and Ullrich, Neuron 9, 383-391, 1992). In contrast, signal transduction by serine/threonine kinase or cytokine receptors often involves formation of multi-component receptor complexes in which the ligand binding and signal transducing components are distinct. Examples are the TGF- receptor complex, a serine/threonine kinase receptor consisting of separate binding (Type II) and signaling (Type I) components and the CNTF family. CNTF, interleukin-6 (IL-6) and leukocyte inhibitory factor (LIF) share the common signaling components, gp130 and/or LIFR, in their respective receptor complexes. While the ligand specificity of these complexes is determined by a specific binding subunit to each individual ligand, signal transduction requires association of the initial complex of ligand and ligand binding subunit with other receptor subunits which cannot bind ligand directly (Ip et al., Cell. 69, 1121-1132, 1992). In the CNTF receptor complex, the ligand binding component is CNTF receptor (CNTFR), which like GDNFR, is a GPI-anchored membrane protein. The present invention involves the description of the first example of a receptor PTK whose autophosphorylation is dependent upon association with a

separate ligand-specific binding component.

The present study confirms that GDNFR-α, a GPI-linked membrane protein that binds to GDNF with high affinity, is required for the efficient association of GDNF with the Ret receptor PTK. In the absence of GDNFR-α, GDNF is unable to bind to Ret or stimulate Ret receptor autophosphorylation. In the presence of GDNFR-α, GDNF associates with Ret and rapidly induces Ret autophosphorylation in a dose-dependent manner. GDNFR-α is able to function in either membrane bound or soluble forms (Figure 11), as discussed above. GDNF concentrations of 50 pg/mL (1.7 pM) are able activate the Ret tyrosine kinase in cells expressing GDNFR-α. This is consistent with the dissociation constant (1.5 pM) found for the high affinity GDNF binding sites on NGR-38 cells. The rapid induction of Ret phosphorylation by GDNF (detectable one minute after treatment) and the ability of Ret-Fc to block autophosphorylation suggest that Ret is being activated directly rather than as a downstream consequence of the phosphorylation of some other receptor.

Cross-linking studies support the hypothesis that efficient association of Ret with GDNF depends on GDNFR-α. Cross-linking of GDNF to Ret in NGR-38 cells which express high levels of GDNFR-α is robust, but in parental Neuro-2a cells cross-linked products are barely detectable. Although conclusive identification of all the cross-linked complexes is difficult, the data clearly demonstrates an association of Ret with GDNF that is dependent on the presence of GDNFR-α, and demonstrates that GDNFR-α is included in some of the cross-linked products. The reason for the presence of minor cross-linked species in Neuro-2a cells is not clear. While the expression of GDNFR-α mRNA in Neuro-2a cells could not be detected by Northern blot, it is possible that GDNFR-α is expressed at very low levels in these cells.

The fact that Ret can be activated by GDNF in cultured rat embryonic spinal cord motor neurons further demonstrates the biological relevance of the Ret/GDNF interaction. These cells are a primary target of GDNF *in vivo*, and have been shown to respond to low doses of GDNF *in vitro* (Henderson et al., 1994). Stimulation of Ret phosphorylation was abolished when the motor neuron cells were pre-treated with PI-PLC (data not shown), suggesting that the activation of Ret by GDNF requires GDNFR-α.

Although binding of ligand to the receptor extracellular domain is the first step in the activation of other known receptor PTKs, the present data has shown that this is not the case for GDNF and Ret. Figure 13 depicts a model for the binding of GDNF to GDNFR-α and Ret, and the consequent activation of the Ret PTK in response to GDNF. The initial event in this process is the binding of disulfide-linked dimeric GDNF to GDNFR-α in either monomeric or dimeric form. Although there is

currently no direct evidence for the existence of dimeric GDNFR-α, when 293T cells were transfected with GDNFR-α cDNA, two classes of binding sites appeared. The simplest explanation for this observation is the existence of monomeric and dimeric GDNFR-α, each with its own ligand binding affinity. This is consistent with the finding that GDNF binding affinities are apparently unaffected by the presence of Ret. Since the present experiments do not address the question of whether dimeric GDNFR-α is in equilibrium with its monomer in the absence of GDNF or if dimerization is induced by GDNF binding, these possibilities are presented as alternate pathways. The complex consisting of dimeric GDNFR-α and dimeric GDNF can bind two molecules of Ret, forming the active signaling complex. As for other PTKs, close contact between the intracellular catalytic domains of two Ret molecules is likely to result in receptor autophosphorylation. This notion that Ret functions by this mechanism is supported by the fact that the MEN2A mutation which causes steady state dimerization of Ret results in constituitive activation of the Ret kinase (Santoro et al., 1995).

Motor neurons have been reported to respond to GDNF with an ED_{50} of as low as 5 fM (Henderson et al., 1994). Although it is difficult to compare binding affinity with the ED_{50} for a biological response, it is possible that very high affinity GDNF binding sites exist on these cells. Other cells, such as embryonic chick sympathetic neurons, have been reported to bind GDNF with a Kd of 1-5 nM (Trupp et al., Journal Of Cell Biology. 130, 137-148, 1995). It is unlikely that GDNFR-α is involved in a receptor complex for such low affinity sites, but a weak direct interaction between GDNF and Ret may be present.

Expression of c-ret has been observed during embryogenesis in many cell lineages of the developing central and peripheral nervous systems, including cells of the enteric nervous system (Pachnis, et al., Development, 119, 1005-1017, 1993; Tsuzuki et al., 1995). Outside the nervous system, c-ret expression has been detected in the Wolffian duct, ureteric bud epithelium and collecting ducts of the kidney (Pachnis, et al., supra; Tsuzuki et al., 1995). Ret expression has also been detected in all neuroblastoma cell lines derived from the neural crest (Ikeda et al., 1990) and from surgically resected neuroblastomas (Nagao et al., 1990; Takahashi & Cooper, 1987). GDNF expression has been observed in both CNS and PNS, as well as in non-neuronal tissues during embryonic development. The levels of GDNF expression found in many non-neuronal tissues were higher than in the nervous system (Choi-Lundberg and Bohn, Brain Res. Dev. Brain Res. 85, 80-88, 1995). Although expression of GDNFR-α has not been extensively studied, primary Northern blot analysis detected the presence of high levels of the GDNFR-α mRNA in the liver,

brain, and kidney of adult rat and mouse. The similarity of the expression patterns of ret, GDNF, and GDNFR-α in developing nervous system and kidney is consistent with their combined action during development.

Mammalian kidney development has been postulated to result from reciprocal interactions between the metanephron and the developing ureter, a branch developed from the caudal part of the Wolffian duct (Saxen, Organogenesis of the kidney. Development and Cell Biology series, Cambridge University Press, Cambridge, England, 1987). While the expression of Ret has been found at the ureteric bud but not in the surrounding mesenchyme in developing embryos, the expression of GDNF was detected in the undifferentiated but not adult metanephric cap of the kidney. These observations suggest that an interaction between GDNF and Ret is responsible for initiating the development of the ureteric structure. Further support for this hypothesis is provided by targeted disruptions of the GDNF and ret genes, which result in very similar phenotypic defects in kidney (Schuchardt et al., Nature. 367, 380-383, 1994; Sanchez, in press). Another major phenotypic defect observed in both GDNF (-/-) and ret (-/-) knockout animals is a complete loss of the enteric neurons throughout the digestive tract. Hirschsprung's disease, a genetic disorder characterized by the congenital absence of parasympathetic innervation in the lower intestinal tract, has also been linked to "loss-of-function" mutations in ret (Romeo et al., Nature. 367, 377-378, 1994. Edery et al., 1994). A later report (Angrist et al., Hum. Mol.Genet. 4, 821-830, 1995) indicated that, contrary to earlier observations, some Hirschsprung's patients do not carry mutations in ret. It is now envisioned that such patients may carry mutations in GDNF, GDNFR-α or some other critical component of this signaling pathway.

Experimental Procedures

[^{125}I]GDNF Binding to Neuro-2a Cells Expressing GDNFR-α

Neuro-2a cells (ATCC #CCL 131) were transfected with an expression plasmid, as described above, using the Calcium Phosphate Transfection System (GIBCO/BRL) according to the manufacturer's directions. Transfected cells were selected for expression of the plasmid by growth in 400 μg/mL G418 antibiotic (Sigma). G418 resistant clones were expanded and assayed for GDNFR-α expression by binding to [^{125}I]GDNF (Amersham, Inc., custom iodination, catalog #IMQ1057). Cells from each clone were seeded at a density of 3 x 10^4 cells/cm^2 in duplicate wells of 24-well tissue culture plates (Becton Dickinson) pre-coated with polyornithine. Cells were washed once with ice-cold washing buffer (DMEM

containing 25 mM HEPES, pH 7.5) and were then incubated with 50 pM [^{125}I]GDNF in binding buffer (washing buffer plus 0.2% BSA) at 4°C for four hours either in the presence or absence of 500 mM unlabeled GDNF. Cells were then washed four times with ice-cold washing buffer, lysed in 1 M NaOH, and the cell-associated radiolabel quantitated in a 1470 Wizard Automated Gamma Counter (Wallac Inc.). The amount of GDNFR-α expressed by individual clones was estimated by the ratio of [^{125}I]GDNF bound to cells in the absence and presence of unlabeled GDNF. Three clones were chosen as representatives of high, moderate, and low level expressors of GDNFR-α for use in binding experiments. The ratios [^{125}I]GDNF bound in the absence and presence of unlabeled GDNF for these clones were: NGR-38) 16:1, NGR-16) 12.8:1, and NGR-33) 8:1. Equilibrium binding of [^{125}I]GDNF to NGR-38 cells was carried out as described above except that concentrations of labeled GDNF ranged from 0.5 pM to 1 nM. In all assays, nonspecific binding as estimated by the amount of radiolabel binding to cells in the presence of 500 nM unlabeled GDNF was subtracted from binding in the absence of unlabeled GDNF. Binding data was analyzed by Scatchard plot.

Chemical Cross-Linking

Neuro-2a or NGR-38 cells were washed once with phosphate-buffered saline (PBS, pH 7.1), then treated for four hours at 4°C with 1 or 3 nM [^{125}I]GDNF in binding buffer in the presence or absence of 500 nM unlabeled GDNF. Following binding, cells were washed four times with ice-cold washing buffer and incubated at room temperature for 45 minutes with 1 mM bis suberate (BS^3, Pierce) in washing buffer. The cross-linking reaction was quenched by washing the cells three times with Tris-buffered saline (TBS, pH 7.5). The cells were then either lysed directly in SDS-PAGE sample buffer (80 mM Tris HCl [pH 6.8], 10% glycerol, 1% SDS, 0.025% bromophenol blue) or in Triton X-100 lysis buffer (50 mM Hepes, pH 7.5, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 1% aprotinin (Sigma, Cat.# A-6279), 1 mM PMSF (Sigma, Cat.# P-7626), 0.5 mM Na_3VO_4 (Fisher Cat.# S454-50). The lysates were clarified by centrifugation, incubated with 5 μg/mL of anti-Ret antibody (Santa Cruz Antibody, C-19, Cat. #SC-167), and the resulting immunocomplexes were collected by precipitation with protein A-Sepharose CL-4B (Pharmacia). The immunoprecipitates were washed three times with the lysis buffer, once with 0.5% NP-40 containing 50 mM NaCl and 20 mM Tris-Cl, pH 7.5, and were then resuspended in SDS-PAGE sample buffer. Both the whole cell lysates and the immunoprecipitates were fractionated by 7.5% SDS-PAGE with a ratio of Bis:Acrylamide at 1:200.

Western Blot Analysis

The autophosphorylation of Ret receptor was examined by Western blot analysis. Briefly, cells were seeded 24 hours prior to the assay in 6-well tissue culture dishes at a density of 1.5 x 10^6 cells /well. Cells were washed once with binding buffer and treated with various concentrations of different reagents (including GDNF, PI-PLC, PI-PLC/CM, and Ret-Fc fusion protein), either alone or in combination, in binding buffer for various periods of times. Treated cells and untreated controls were lysed in Triton X-100 lysis buffer and immunoprecipitated with the anti-Ret antibody (Santa Cruz, C-19, Cat. #SC-167) and protein-A Sepharose as described above. Immunoprecipitates were fractionated by SDS-PAGE and transferred to nitrocellulose membranes as described by Harlow and Lane (Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1988). The membranes were pre-blocked with 5% BSA (Sigma) and the level of tyrosine phosphorylation of the receptor was determined by blotting the membrane with an anti-phosphotyrosine monoclonal antibody 4G10 (UBI, Cat. #05-321) at room temperature for two hours. The amount of protein included in each lane was determined by stripping and re-probing the same membrane with the anti-Ret antibody. Finally, the membrane was treated with chemiluminescence reagents (ECL, Amersham) following the manufacturer's instructions and exposed to X-ray films (Hyperfilm-ELC, Amersham).

Treatment of Cells with PI-PLC and Generation of PI-PLC Treated Conditioned Media

In order to release GPI-linked GDNFR-α from the cell surface, cells were washed once with washing buffer, then incubated with 1 U/mL phosphatidylinositol specific phospholipase C (PI-PLC, Boehringer Mannheim, Cat. #1143069) in binding buffer at 37°C for 45 minutes. The cells were then washed three times with washing buffer and further processed for Ret autophosphorylation assay or cross-linking. For generation of PI-PLC treated conditioned media (PI-PLC/CM), 8 x 10^6 cells were removed from tissue culture dishes by treating the cells with PBS containing 2 mM of EDTA at 37°C for 5 to 10 minutes. Cells were washed once with washing buffer, resuspended in 1 mL of binding buffer containing 1 U/mL of PI-PLC, and incubated at 37°C for 45 minutes. The cells were pelleted, and the PI-PLC/CM was collected.

Preparation of the Ret-Fc Fusion Protein

A cDNA encompassing the entire coding region of c-Ret was isolated from a day 17 rat placenta cDNA library using an oligonucleotide probe corresponding to the

first 20 amino acids of the mouse c-Ret (Iwamoto et al., 1993; van Heyningen, 1994). The region coding for the extracellular domain of the Ret receptor (ending with the last amino acid, R636) was fused in-frame with the DNA coding for the Fc region of human IgG (IgG1) and subcloned into the expression vector pDSR2 as previously described (Bartley et al., Nature. 368, 558-560, 1994). The ret-Fc/pDSRa2 plasmid was transfected into Chinese hamster ovary (CHO) cells and the recombinant Ret-Fc fusion protein was purified by affinity chromatography using a Ni^{++} column (Qiagen).

Preparation of Embryonic Rat Spinal Cord Motor Neuron Cultures

Enriched embryonic rat spinal cord motor neuron cultures were prepared from entire spinal cords of E15 Sprague-Dawley rat fetuses 24 hours before the experiments. The spinal cords were dissected, and the meninges and dorsal root ganglia (DRGs) were removed. The spinal cords were cut into smaller fragments and digested with papain in L15 medium (Papain Kit, Worthington). The motor neurons, which are larger than other types of cells included in the dissociated cell suspension, were enriched using a 6.8% Metrizamide gradient (Camu and Henderson, J Neuroscience. 44, 59-70, 1992). Enriched motor neurons residing at the interface between the metrizamide cushion and the cell suspension were collected, washed, and seeded in tissue culture dishes pre-coated with poly-L-ornithine and laminin at a density of ~9 x 10^4 cells/cm^2 and were cultured at 37°C.

Example 10

GRR2 Mediation of Neurturin and GDNF-Induced Ret Activation

The present study demonstrates that neurturin binds to both GDNFR-α and GRR2, a novel receptor related to GDNFR-α. Both GDNFR-α and GRR2 can mediate neurturin-induced autophosphorylation of the Ret protein tyrosine kinase. GDNF also binds both GDNFR-α and GRR2, and activates Ret in the presence of either binding receptor. However, neurturin binds GRR2 more effectively than GDNF, while GDNF binds GDNFR-α more efficiently than neurturin. These data indicate that, while there is crosstalk, GDNF is the primary ligand for GDNFR-α and neurturin appears to exhibit a preference for GRR2.

Introduction

Recently, Kotzbauer et al. (Nature, 384, 467-470, 1996) reported the cloning of neurturin, a novel neurotrophic factor that is approximately 42% identical in amino acid sequence to GDNF. Both GDNF and neurturin are synthesized in pre-pro forms and their precursor molecules are proteolytically processed to yield mature proteins of about 100 amino acids that assemble into disulfide-linked homodimers. All seven cysteine residues crucial for the structure of GDNF and their spacing patterns are conserved in neurturin (Kotzbauer et al., 1996). Although the biological activities of neurturin have not yet been thoroughly investigated, they appear to be very similar to those of GDNF. Both neurturin and GDNF have been shown to promote the survival of sympathetic neurons derived from the superior cervical ganglia (SCG) and of sensory neurons of both the nodose (NG) and dorsal root ganglia (DRG). Neurturin and GDNF mRNAs are widely distributed in a variety of both neuronal and non-neuronal tissues of embryos and adults. Both are found in brain, kidney, and lung, whereas neurturin mRNA is also expressed at high levels in neonatal blood.

The structural and biological similarities between GDNF and neurturin suggest that their action may be mediated by the same or related receptors. The receptor for GDNF consists of a complex of GDNF receptor α (GDNFR-α) and the Ret protein tyrosine kinase (PTK) (Jing et al., Cell, 85, 1113-1124, 1996; Treanor et al., Nature, 382, 80-83, 1996). GDNFR-α is a glycosyl-phosphodylinositol (GPI) anchored cell surface molecule that serves to bind GDNF but cannot signal independently since it lacks a cytoplasmic domain. GDNF signaling is accomplished via association of the complex of GDNF and GDNFR-α with Ret, resulting in activation of the Ret kinase.

GDNFR-α mRNA is widely distributed in neuronal and nonneuronal tissues and is expressed through embryonic development to adulthood, implying a broad spectrum of biological functions (Treanor et al., 1996; Fox et al., unpublished data). The other component of the GDNF receptor complex, Ret, is a receptor type PTK encoded by the *ret* proto-oncogene. Ret mRNA and protein are highly expressed in the CNS and PNS, as well as in the kidney. Various mutations in the *ret* gene are associated with inherited human diseases, including familial medullary thyroid carcinoma (FMTC), multiple endocrine neoplasia type 2A (MEN2A) and 2B (MEN2B), and Hirschsprung's disease. Targeted disruption of the ret gene in knockout mice results in severe phenotypic defects, including renal agenesis or severe dysgenesis and lack of entire enteric nervous system. These defects are extremely similar to those caused by GDNF null mutations, implying that GDNF-mediated signaling through Ret is required for the development of these tissues. Much less

severe defects, however, were detected in a number of neuronal structures in which both GDNFR-α and Ret are expressed, such as the trigeminal and vestibular ganglia, the facial motor nucleos, the substantial nigra, and the locus coeruleus (Schuchardt et al., Nature, 367, 380-383, 1994; Treanor et al., 1996). This suggests that either GDNF signaling is not required for the embryonic development of these structures, or that some unknown signaling molecules similar to GDNF or Ret may exist that can substitute for them. Alternatively, the embryonic development of these tissues may completely rely on another yet unknown signaling system.

This example describes the cloning of a novel GDNFR-α related receptor, GRR2, and provides evidence that GRR2 is a receptor for neurturin. Analogous to GDNF and GDNFR-α, neurturin effectively binds GRR2 and induces Ret activation. The data also show that both GDNF and neurturin can interact with either GDNFR-α or GRR2 and activate the Ret PTK in the presence of either binding receptor.

Results

Cloning and Sequence Analysis of GRR2

A human expressed sequence tag (EST) with significant homology to GDNFR-α was found by a FASTA search of the publicly available nucleic acid sequence databases (Marra et al., 1996, WashU-HHMI Mouse EST Project, unpublished). Oligonucleotides corresponding to the ends of this EST were synthesized and used in a reverse transcription-polymerase chain reaction (RT-PCR) with human fetal brain mRNA as the template. A fragment of the expected length was isolated and used as a hybridization probe to screen a human fetal brain cDNA library. Five positive clones were identified and the longest clone was sequenced. This clone contained a large open reading frame coding for a 464 amino acid protein related in sequence to GDNFR-α. We have named this protein GDNFR-α Related Receptor 2 (GRR2). The oligonucleotides described above were also used to screen pools from a rat photoreceptor cDNA library (Jing et al., 1996) by PCR and a product of the expected length was obtained from a single pool. An individual cDNA clone from this pool was identified by hybridization to the radiolabeled PCR product and sequenced. This clone contained a 2.2 kb insert with an open reading coding for a 460 amino acid peptide that is nearly identical to human GRR2.

A comparison of the amino acid sequences of human and rat GDNFR-α and GRR2 is shown in Figure 20. Shaded areas indicate amino acid sequence conservation between all four receptors while boxes indicate conservation only between the same receptor from different species. The amino acid sequences of both

GDNFR-α and GRR2 are extremely well-conserved between species, each human receptor being 92% identical to its rat counterpart. The overall amino acid sequence identity between human GDNFR-α (hGDNFR-α) and human GRR2 (hGRR2) is 48%. The sequence is most divergent in the C-terminal region--amino acids 350-465 of hGDNFR-α are only 22% identical to amino acids 361-464 of hGRR2. In the N-terminal region, hGDNFR-α and hGRR2 are more closely related, sharing 56% amino acid identity. The corresponding identities between the rat GDNFR-α and GRR2 (rGDNFR-α and rGRR2) are very similar: 48% overall, 26% in the C-terminal region, and 55% in the N-terminal region. The sequence comparison indicates that GDNFR-α and GRR2 are likely to be structurally very similar. The positions of 30 of the 31 cysteine residues (shown in boldface, Figure 20) found in GDNFR-α are conserved in both human and rat GRR2 (one additional cysteine residue is present near the N-terminus of hGRR2). In addition, the hydrophobic C-terminus involved in GPI-linkage of GDNFR-α to the cell membrane (Jing et al., 1996; Treanor et al., 1996) is also present in GRR2.

Figure 20. Comparison of GDNFR-α And GRR2 Peptide Sequences

The amino acid sequences of human and rat GDNFR-α and GRR2 are aligned. Shaded areas indicate amino acids that are identical in all four sequences. Boxes indicate conservation between rat and human orthologs of the same receptor, but not between GDNFR-α and GRR2.

Both Neurturin And GDNF Bind to LA-N-5 And NGR-38 Cells

LA-N-5 is a human neuroblastoma cell line (Sonnenfeld and Ishii, J. Neuroscience Research, 8:375-391, 1982) that expresses high levels of *ret* mRNA (Bunone et al., Exp. Cell. Res., 217:92-99, 1995). RT-PCR experiments using primers specific to GDNFR-α and GRR2 showed that these cells express GRR2 mRNA, but GDNFR-α mRNA was not detected (data not shown). NGR-38 is a cell line derived from mouse Neuro-2a cells (Jing et al., 1996). It expresses high levels of both GDNFR-α and Ret (Jing et al., 1996), but no detectable GRR2 (data not shown), and binds GDNF specifically. LA-N-5 and NGR-38 cells were incubated with $[^{125}I]$-labeled recombinant human neurturin (NTN) or GDNF in the absence or presence of excess unlabeled ligand. As shown in Figure 21A, $[^{125}I]$NTN bound to LA-N-5 cells more strongly than $[^{125}I]$GDNF, although both bound at detectable levels. The binding of $[^{125}I]$NTN to LA-N-5 cells was significantly inhibited by unlabeled neurturin, but not by GDNF. $[^{125}I]$GDNF also bound to LA-N-5 cells, however, the binding was inhibited by either cold GDNF or neurturin.

Figure 21B depicts the binding of [^{125}I]NTN and [^{125}I]GDNF to the GDNFR-α expressing cell line NGR-38. Although both [^{125}I]NTN and [^{125}I]GDNF bound to NGR-38 cells, [^{125}I]GDNF bound more strongly. As was observed for LA-N-5 cells, the binding of [^{125}I]GDNF to NGR-38 cells was inhibited by both unlabeled neurturin and GDNF, while binding of [^{125}I]NTN was only replaceable by neurturin (Figure 21B).

Figure 21. Binding of Neurturin and GDNF to LA-N-5 and NGR-38 Cells

LA-N-5 (A) and NGR-38 (B) cells were incubated with 50 pM of either [^{125}I]NTN or [^{125}I]GDNF in the absence (light gray bars) or presence of unlabeled GDNF (dark gray bars) or neurturin (black bars) at 4°C for 2 hours. The unbound ligands were removed at the end of the incubation and the radioactivity associated with the cells was determined as described.

Cross-Linking of Neurturin and GDNF to GDNFR-α and GRR2

The binding experiments suggest that both neurturin and GDNF interact with GDNFR-α and GRR2. However, lack of a GRR2 specific antibody made further study of these interactions difficult. To overcome this difficulty, plasmids were generated that transiently express GDNFR-α/Fc and GRR2/Fc fusion proteins when transfected into 293T cells. Conditioned medium (CM) containing either GDNFR-α/Fc or GRR2/Fc fusion proteins was incubated with [^{125}I]NTN or [^{125}I]GDNF, chemically cross-linked, and then precipitated directly using Protein-A Sepharose beads. The immunoprecipitates were analyzed by SDS-PAGE (Figure 22). Major species of 100-120 kD and 90-110 kD were observed when [^{125}I]GDNF or [^{125}I]NTN were used, respectively (Figure 22). Strong bands with higher molecular mass, ~300 kD for GDNFR-α/Fc and ~280 kD for GRR2/Fc, were also observed (Figure 22). In addition, minor bands of ~15 kD, 35 kD, and 60 kD in the [^{125}I]GDNF lanes and ~12 kD, 26 kD, and 50 kD in the [^{125}I]NTN lanes, were visible (Figure 22). When CM from mock transfected cells were used, no cross-linked band was precipitated by Protein-A Sepharose (data not shown). None or much weaker radio-labeled bands were detected when excess unlabeled ligands were added in the control samples (Figure 22).

Figure 22. Chemical Cross-Linking of Neurturin And GDNF to GDNFR-α and GRR2 Receptors.

CM containing GDNFR-α/Fc (GDNFR-α) or GRR2/Fc (GRR2) fusion proteins were incubated with either 10 nM of [^{125}I]NTN (N) or 5 nM of [^{125}I]GDNF (G) in the presence (+ unlabeled) or absence (- unlabeled) neurturin (N) or GDNF (G). The bound receptor-ligand complexes were chemically cross-linked by 1 mM of BS^3, precipitated with Protein-A Sepharose and analyzed by SDS-PAGE as described. The solid arrow indicates the 90-110 kD and the 100-120 kD cross-linked species. The open arrow depicts the ~280 kD and ~300 kD complexes.

Neurturin Induces Ret Autophosphorylation in Cells That Express GDNFR-α

The ability of neurturin to associate with GDNFR-α indicates that neurturin, like GDNF, may activate Ret through GDNFR-α. In order to examine this possibility, the ability of neurturin to induce Ret autophosphorylation in NGR-38 cells was tested. NGR-38 cells were treated with concentrations of neurturin ranging from 0 to 50 nM, lysed, and the lysates immunoprecipitated with anti-Ret antibody. The immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting using an anti-phosphotyrosine antibody. A 170 kD band, indicating autophosphorylation of tyrosine residues on the mature form of Ret, was observed in all lanes (Figure 23, lanes 8-14 from left). A much weaker corresponding band was observed in neurturin-treated Neuro-2a cells (data not shown). The induction of Ret autophosphorylation by neurturin was dose-dependent. Stimulation of Ret autophosphorylation in NGR-38 cells could be detected with 500 pM neurturin (Figure 23). In a parallel experiment using GDNF in place of neurturin, an increase in the level of phosphorylation of the 170 kD Ret band over background could be seen at a GDNF concentration of 5 pM (Figure 23, lanes 1-7 from left). When the filters were stripped and re-probed with the anti-Ret antibody, the 170 kD Ret protein band appeared in all lanes with approximately equal intensity (data not shown).

Figure 23. Neurturin and GDNF Induce Ret Autophosphorylation in NGR-38 Cells

NGR-38 cells were treated with various concentrations of GDNF or neurturin as described. The cells were lysed, immunoprecipitated with anti-Ret antibody, fractionated by SDS-PAGE, and blotted with anti-phosphotyrosine antibody for Ret phosphorylation. The bands of phosphorylated Ret are indicated by an arrow.

Neurturin And GDNF Induce Ret Autophosphorylation in LA-N-5 Cells

Both neurturin and GDNF bind to GRR2, and the Ret PTK can be activated by either neurturin or GDNF through GDNFR-α. These observations suggest that GRR2 may also be able to mediate neurturin and/or GDNF activation of Ret. To assess this possibility, human LA-N-5 neuroblastoma cells expressing GRR2 and Ret were treated with various concentrations of neurturin or GDNF and processed for immunoblotting as described in the previous section (Figure 24). As shown, both neurturin and GDNF induced Ret autophosphorylation (Figure 24).

Figure 24. Neurturin And GDNF Induced Ret Autophosphorylation in LA-N-5 Cells

LA-N-5 cells were treated with various concentrations of GDNF or neurturin as described. The cells were lysed, immunoprecipitated with anti-Ret antibody, fractionated by SDS-PAGE, and blotted with anti-phosphotyrosine antibody for Ret phosphorylation. The bands of phosphorylated Ret are indicated by an arrow.

Neurturin And GDNF Induce MAP Kinase activation in LA-N-5 And NGR-38 Cells

We have demonstrated that both neurturin and GDNF can induce Ret autophosphorylation in cells expressing either GDNFR-α or GRR2. We then tested if the activation of Ret kinase by neurturin and/or GDNF could lead to activation of the downstream signaling molecule MAP kinase. Both LA-N-5 and NGR-38 cells were treated with either neurturin, GDNF, or NGF. Treated cells were lysed directly in SDS-PAGE sample buffer, fractionated by SDS-PAGE, and immunoblotted using an anti-phosphorylated MAP kinase antibody (New England Biolabs, Beverly, MA). As shown in Figure 25, both p44 and p42 isoforms of MAP kinase are apparently activated by both neurturin and GDNF in either LA-N-5 or NGR-38 cells. MAP kinase activation by NGF (used as a positive control) was also observed.

Figure 25 (Panels A and B). Neurturin And GDNF Induced MAP Kinase Activation in LA-N-5 And NGR-38 Cells

25A. LA-N-5 cells were treated with various concentrations of GDNF or neurturin as described. The cells were lysed directly in 2 X SDS-PAGE sample buffer containing 0.5 mM $NaVO_4$, fractionated by SDS-PAGE, and blotted with an antibody against phosphorylated MAP kinase (MAPK-P). 25B. The membrane was stripped and reprobed with an anti-MAP kinase antibody for the amount of MAP kinase proteins loaded in each lane (MAPK).

Discussion

Signal transduction by most receptor PTKs starts by direct interaction with their ligands and consequent activation of the receptors. Cloning and characterization of GDNFR-α, an accessory molecule for ligand binding, revealed a novel mechanism by which Ret receptor PTK transduces the GDNF signal. GDNF does not bind Ret alone, instead, it first binds to GDNFR-α and then interacts with Ret as a part of the GDNF-GDNFR-α complex. The newly cloned GRR2 is related to GDNFR-α at both the amino acid level and the three dimensional structure. It shares 48% identical amino acid residues with GDNFR-α, among which are 30 of the 31 cysteines.

We have demonstrated that both neurturin and GDNF bind to GDNFR-α and GRR2. Binding of GDNF or neurturin to either GDNFR-α or GRR2 results in further association of the ligand with Ret and consequent activation of the Ret PTK and the MAP kinase, a downstream signaling molecule. However, each of the ligands appears to bind to one receptor preferentially. Neurturin binds GRR2 expressing LA-N-5 cells more efficiently than GDNF, and GDNF binds GDNFR-α expressing NGR-38 cells more efficiently than neurturin. It is not clear at this time why the binding of [125I]GDNF to both GDNFR-α and GRR2 can be replaced by both unlabeled GDNF and neurturin, but that of [125I]NTN can only be inhibited by cold neurturin.

Consistent with the binding study, GDNF is more effectively cross-linked to GDNFR-α/Fc fusion receptors than to GRR2/Fc, while neurturin cross-linking shows the opposite result.

Experimental Procedures

cDNA Cloning of GRR2

A search of the GenBank database for sequences related to GDNFR-α resulted in the identification of EST, H12981.Gb_Est1. Primers corresponding to nucleotides 47 to 65 (5'-CTGCAAGAAGCTGCGCTCC-3') and 244 to 265 (5'-CTTGTCCTCATAGGAGCAGC-3') of H12981.Gb_Est1 were synthesized and used for RT-PCR with human fetal brain mRNA (Clontech, Cat. #64019-1) as the template. A 218 nt fragment was amplified, subcloned into pBlue-Script (Stratagene, La Jolla, CA), and sequenced to verify its correspondence with the original EST. The fragment was then radiolabeled with [^{32}P]-dCTP using a Random Primed DNA Labeling Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The radio-labeled probe was used to screen a human fetal brain cDNA library (Stratagene, La Jolla, CA). Two million clones were plated on 15 cm agarose plates and replicated on

duplicate nitrocellulose filters. The filters were prehybridized at 55°C for 3.5 hours in 200 ml of 6 x SSC, 1 x Denhardts, 0.5% SDS, and 50 µg/ml salmon sperm DNA. Following the addition of 2 x 10^8 cpm of the radiolabeled probe, hybridization was continued for 18 hours. Filters were then washed twice for 30 minutes each at 55°C in 0.2 x SSC, 0.1% SDS and exposed to X-ray film overnight with an intensifying screen. Five positive clones were identified and their DNA sequences were determined.

The oligonucleotide primers described above were also used for PCR screening of DNAs isolated from 27 pools (1500 clones each) of a rat photoreceptor cDNA library (Jing et al., 1996). A single positive pool was identified and screened by hybridization to the same radio-labeled probe as described above. An individual cDNA clone from this pool was identified and sequenced.

<u>DNA Sequencing and Sequence Analysis</u>

DNA sequencing was performed using an automated Applied Biosystems 373A DNA sequencer and Taq DyeDeoxy Terminator cycle sequencing kits (Applied Biosystems, Foster City CA). Comparison of the GDNFR-α and GRR2 sequences with public databases was carried out using the FASTA computer algorithm (Pearson and Lipman, Proceedings Of The National Academy Of Sciences Of The United States Of America. 85, 2444-2448, 1988). The peptide sequences of GDNFR-α and GRR2 were aligned using the Lineup program. All sequence analysis programs used were included in the Wisconsin sequence analysis package (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, WI).

<u>Binding of [^{125}I]GDNF and [^{125}I]NTN to NGR-38 and LA-N-5 Cells</u>

Recombinant human neurturin was expressed in E. coli as insoluble protein. The inclusion bodies were solubilized, and the neurturin protein was re-folded and purified by ion exchange and hydrophobic interaction chromatography.

[^{125}I]NTN (~2000 Ci/mmole) was prepared using purified E. coli expressed protein (Amersham, Inc. , Arlington Heights, IL; custom iodination, catalog #IMQ1057). Recombinant human GDNF was also radio-iodinated (Jing et al., 1996). Binding of [^{125}I]NTN and [^{125}I]GDNF to LA-N-5 and NGR-38 cells were carried out as previously described (Jing et al., 1990). Briefly, cells were seeded one day before the assay in 24-well Costar tissue culture plates pre-coated with polyornithine at a density of 3 x 10^4 cells/cm^2. Cells were placed on ice for 5 to 10 minutes, washed once with ice-cold buffer (DMEM containing 25 mM HEPES [pH 7.0]) and incubated

at 4°C in 0.2 ml binding buffer (washing buffer containing 2 mg/ml bovine serum albumin) containing various concentrations of [^{125}I]NTN or [^{125}I]GDNF in the absence or presence of 500 nM unlabeled ligands for 4 hours. Cells were washed 4 times with 0.5 ml ice-cold washing buffer and lysed with 0.5 ml of 1 M NaOH. The lysates were counted in a 1470 Wizard Automatic Gamma Counter (Wallac Inc., Gaithersburg, MD).

Chemical Cross-Linking

The coding regions of the first 455 amino acids of human GDNFR-α and the first 451 residues of human GRR2 cDNAs were fused in frame with a DNA fragment encoding the Fc region of human IgG1 tagged with 6 histidine residues at the carboxy terminus (Culouscou et al., J. Biochem., 270:12857-12863, 1995). This construct was then inserted into the expression vector pBK RSV (Stratagene, La Jolla, CA) as previously described (Jing et al., 1996). The GDNFR-α/Fc and GRR2/Fc fusion constructs were transfected into 293T cells, and conditioned media (CM, DMEM supplied with 0.5% fetal calf serum) containing the fusion proteins were collected 4 days after transfection. Aliquots of 1 ml CM plus 50 µl of 1 M HEPES, pH 7.5 were incubated at 4°C with 10 nM of [^{125}I]NTN or 5 nM [^{125}I]GDNF in the presence or absence of 1 µM of unlabeled ligand for 4 hours. Bis suberate (BS^3 Pierce, Rockford, IL) stock solution in washing buffer (40 mM) was added to each binding mixture to a final concentration of 1 mM, mixed and incubated at room temperature for 30 minutes. The reaction was quenched by adding 50 µl of 1 M glycine and incubating at room temperature for 15 minutes. Triton X-100 was added to a final concentration of 1%, and the cross-linked product was precipitated directly with 200 µl of Protein-A Sepharose CL-4B (Pharmacia). The cross-linked products were analyzed by 7.5% SDS-PAGE under reducing conditions.

Immunoblotting Analysis

Ret autophosphorylation was examined by immunoblot analysis as previously described (Jing et al., 1996). Briefly, cells were seeded 24 hours prior to the assay in 6-well tissue culture dishes at a density of 1.5 x 10^6 cells /well. Cells were washed once with binding buffer and treated with various concentrations of neurturin or GDNF (0.5 pM - 50 nM) in binding buffer at 37°C for 10 minutes. Treated cells and untreated controls were lysed in Triton X-100 lysis buffer (50 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 1% aprotinin (Sigma, Cat.# A-6279), 1 mM PMSF (Sigma, Cat.# P-7626), 0.5 mM

Na_3VO_4 (Fisher Cat.# S454-50) and immunoprecipitated with an anti-Ret antibody (Santa Cruz Biotechnology) and protein-A Sepharose as described (Jing et al., 1996). Immunoprecipitates were fractionated by 7.5% SDS-PAGE and transferred to nitrocellulose membranes as described by Harlow and Lane (Antibodies LAboratory Manual, Spring Harbor Laboratory, Spring Harbor Press, 1988). The membranes were blocked with 5% BSA (Sigma) and tyrosine phosphorylation of the Ret receptor was detected by probing with an anti-phosphotyrosine monoclonal antibody 4G10 (UBI, Cat #05-321) at room temperature for 2 hours. The amount of Ret protein in each lane was determined by stripping and re-probing the same membrane with the anti-Ret antibody. Detection was accomplished using a sheep anti-mouse secondary antibody or protein-A conjugated to horseradish peroxidase (Amersham, cat.#NA931) in conjunction with chemiluminescence reagents (ECL, Amersham) following the manufacturer's instructions.

Activation of the MAP kinases was analyzed using a PhosphoPlus MAPK Antibody Kit (New England Biolabs, Beverly, MA, Cat. #9100) following manufacturer's instructions. LA-N-5 and NGR-38 cells were seeded in 6-well dishes as described above. Cells were quiesced in DMEM containing 0.5% fetal calf serum (FCS) at 37°C for 24 hours. The cells were then incubated with fresh media for 2 hours, treated with 50 ng/ml of NGF, GDNF, or neurturin at 37°C for 5 minutes, and lysed directly in 150 µl of 2 X SDS-PAGE sample buffer containing 0.5 mM NaVO4. The cell lysates were fractionated by 10% SDS-PAGE and transferred to a nitrocellulose filter. The filter was blocked with 5% non-fat dry milk at 4°C overnight and then incubated overnight at 4°C with a 1:1000 dilution of anti-phosphorylated MAP kinase antibody in the same buffer (New England Biolabs). Bands were detected using a horseradish peroxidase conjugated anti-rabbit antibody and the LumiGLO chemiluminescent reagents according to the manufacturer's recommendations. After exposure to X-ray film, the filter was stripped and reprobed by the anti-MAPK antibody .

Figure 25 (Panels A and B). Neurturin And GDNF Induced MAP Kinase Activation in LA-N-5 And NGR-38 Cells

25A. LA-N-5 cells were treated with various concentrations of GDNF or neurturin as described. The cells were lysed directly in 2 X SDS-PAGE sample buffer containing 0.5 mM $NaVO_4$, fractionated by SDS-PAGE, and blotted with an antibody against phosphorylated MAP kinase (MAPK-P). 25B. The membrane was stripped and reprobed with an anti-MAP kinase antibody for the amount of MAP kinase proteins loaded in each lane (MAPK).

Example 11

Cloning and Expression of GRR2 and GRR3

Signaling by glial cell line-derived neurotrophic factor (GDNF) is mediated by two receptor components. GDNF receptor-α (GDNFR-α) binds GDNF specifically, leading to the association of GDNF with Ret and the activation of the Ret kinase. Similarly, neurturin induces Ret activation through association with GRR2, a GDNFR-α-related receptor. Both GDNFR-α and GRR2 are capable of binding either GDNF or neurturin, but each exhibits a marked preference for its cognate ligand. A third molecule was cloned and is related in structure and primary amino acid sequence to GDNFR-α and GRR2. This molecule has been named GDNFR-α-related receptor 3 (GRR3). Analysis of the tissue distribution of GDNFR-α, GRR2, GRR3, and Ret by mRNA blot and *in situ* hybridization reveals overlapping but distinct patterns of expression. Consistent with their role in GDNF function, GDNFR-α and *ret* are co-expressed at known sites of GDNF action. GRR2 and GRR3 transcripts are also co-localized with those of *ret* in some cases, suggesting that GRR3 may also mediate Ret activation by GDNF or a related ligand.

Introduction

Glial cell line-derived neurotrophic factor (GDNF) is a potent survival factor for midbrain dopaminergic neurons, motor neurons, and several other types of neuronal cells. Targeted disruption of the GDNF gene in mice causes complete renal agenesis and the absence of enteric neurons (Moore et al., Nature, 382, 76-79, 1996; Pichel et al., Nature, 382, 73-76, 1996; Sanchez et al., Nature, 382, 70-73, 1996; and Hudson et al., Brain Research Bulletin, 36, 425-32, 1995), indicating an essential role for GDNF in the development of the renal and the enteric nervous systems. The GDNF receptor was discovered to consist of a novel ligand binding component, GDNFR-α, and a signaling component, the Ret receptor protein tyrosine kinase.

GDNFR-α is attached to the cell membrane through a glycosyl-phosphatidylinositol (GPI) linkage but has no cytoplasmic domain. It binds GDNF specifically and with high affinity regardless of whether or not Ret is present. Ret is a receptor protein tyrosine kinase (PTK) originally discovered as a large open reading frame in the *ret* proto-oncogene. Its unique extracellular domain structure, which includes a signal peptide, a cadherin-like motif, and a cysteine-rich region, places it

outside any other known receptor PTK sub-family. Ret alone does not bind GDNF, but was found to form a complex with GDNF and GDNFR-α that results in Ret activation. Activation of the Ret kinase appears to be associated with the biological effects of GDNF. Targeted disruption of the Ret PTK gene results in a phenotype nearly identical to that resulting from the disruption of GDNF (Schuchardt et al., Nature, 367, 380-383, 1994). *In situ* hybridization and immunohistochemical analysis detects high level expression of *ret* mRNA and protein in the developing central and peripheral nervous systems and in the excretory system of the mouse embryo. This expression pattern is similar to that of GDNF and is consistent with Ret's role in GDNF signaling.

The expression pattern of GDNFR-α is also consistent with its involvement in GDNF signaling. GDNFR-α mRNA has been found in a number of GDNF-responsive cell types and structures of the nervous system, often colocalized with *ret*. In the central nervous system, GDNFR-α mRNA has been observed in both developing and adult rat ventral midbrain, facial nucleus and ventral spinal cord. In addition, some specific cells in the superior colliculus, the lateral septum, the molecular layer of cerebellum adjacent to Purkinje cells, and some nuclei in cerebral cortex and the dorsomedial tegmental area have been shown to express GDNFR-α. In the peripheral nervous system, GDNFR-α mRNA expression has been found in subpopulations of neurons in dorsal root ganglia, in enteric neurons, and in neurons from sympathetic ganglia. High levels of GDNFR-α mRNA expression were also observed in other regions of the nervous system, including the retina, thalamus, pons, and medulla oblongata. Expression has also been seen in non-neuronal tissues such as the developing nephrons, pituitary, urogenital tract and pancreatic primordium.

Neurturin is a molecule which has similarities to GDNF in both amino acid sequence and biological activity. The GRR2 protein (GDNFR-α-Related Receptor 2), is a novel protein related in amino acid sequence to GDNFR-α. GRR2 is capable of binding both GDNF and neurturin, and like GDNFR-α, mediates the activation of the Ret PTK in response to these ligands. Although both GDNF and neurturin can bind both GDNFR-α and GRR2, GDNF exhibits a marked preference for GDNFR-α while neurturin interacts more strongly with GRR2. GDNFR-α-Related Receptor 3 (GRR3) a third member of this receptor family has also been found. The present study examines the tissue and cell-specific mRNA expression of GDNFR-α, GRR2, GRR3, and *ret*.

Results

Molecular Cloning and Sequence Comparison of GRR3 with GRR2 and GDNFR-α

Examination of publicly available sequence databases revealed the presence of a short expressed sequence tag (EST) with sequence homology to the GDNFR-α and GRR2 cDNA clones (WashU-HHMI Mouse EST Project). Oligonucleotides corresponding to the ends of this EST were used as primers in a reverse transcription-polymerase chain reaction (RT-PCR) with total rat embryo RNA as the template. A 225 nucleotide (nt) fragment was amplified, cloned into a plasmid vector, and sequenced to verify that it corresponded to the original GDNFR-α/GRR2-related EST. Plasmid DNAs isolated from pools of an E15 rat embryo cDNA library were screened by PCR and a single positive pool was found. Clones from this pool were screened by hybridization to the radiolabeled 225 nt PCR fragment and a single positive clone was isolated. Sequence analysis of the 1.8 kb insert from this clone revealed an open reading frame coding for a 397 amino acid peptide related to both GDNFR-α and GRR2. This protein was designated GDNFR-α-related receptor 3 (GRR3).

An alignment of the amino acid sequences of rat GDNFR-α, GRR2, and GRR3 is shown in Figure 26. (SEQ ID NO:44) The overall amino acid sequence identity among the three receptors is in the range of 30%-50%. GDNFR-α and GRR2 are somewhat more closely related to each other (48% identity) than they are to GRR3 (35% and 33% identity, respectively). Hydrophobic regions are found at both the amino and carboxy termini of all three molecules, except for the amino terminus of GRR2 (underlined, Figure 26). The amino terminal regions of both GDNFR-α and GRR3 have the characteristics expected for signal peptide sequences. Although the GRR2 N-terminal sequence does not fit the criteria for a classical signal peptide, there is evidence that GRR2 is secreted. The carboxy terminal hydrophobic region of GDNFR-α is known to be involved in GPI-linkage to the cell membrane, and it is likely that the corresponding regions in GRR2 and GRR3 serve the same purpose. The most striking feature of the sequence alignment is the conservation of 28 cysteine residues among all three receptors (highlighted, Figure 26), (SEQ ID NO:44) indicating that these proteins probably have similar three-dimensional structures. Several potential N-glycosylation sites are present in the receptors (shown in boldface, Figure 26), but none are found at the same position in all three receptors. GDNFR-α and GRR2 share sites at positions 365 and 427 that are not found in GRR3, and GRR2 shares a possible site with GRR3 at positions 322-323 (Figure 26).

Expression of GDNFR-α, GRR2, and GRR3 in Adult Rat

The expression of GDNFR-α, GRR2 and GRR3 mRNAs in adult rat tissues was examined by blot hybridization analysis. GDNFR-α mRNA is widely expressed, with high levels found in lung, brain, liver, kidney and spleen. Expression is also detectable in heart and among the tissues examined is absent only in muscle and testis. Two distinct size transcripts are observed and their relative amounts vary among the tissues. The 3.6 kb transcript is predominant in liver, lung, heart, and spleen while comparable amounts of the 3.6 kb and 8.5 kb transcripts are present in brain and kidney. The tissue distribution of GRR2 mRNA is similar to that of GDNFR-α. GRR2 expression is highest in lung, spleen and brain, with lesser amounts in kidney and heart. One difference is the lack of GRR2 expression in liver. The size of the GRR2 transcripts is approximately 3.6 kb, similar to the smaller of the two GDNFR-α transcripts. The expression of GRR3 mRNA is highest in kidney and is absent in brain. Detectable expression of GRR3 is also present in spleen, lung, liver, and heart. The transcript size for GRR3 is somewhat smaller (~2.1 kb) than that observed for GDNFR-α and GRR2.

Expression of GDNFR-α, GRR2 and GRR3 in Mouse Embryo

Developmental expression of GDNFR-α, GRR2, and GRR3 mRNA was examined in the mouse on embryonic days 7, 11, 15, and 17. Expression of the 3.6 kb transcript of GDNFR-α is first apparent at E11, seems to decrease somewhat at E15, but then increases dramatically by E17. A minor amount of the 8.5 kb GDNFR-α mRNA can be detected on E11, but no expression of this transcript is detected thereafter. The expression of the 3.6 kb GRR2 transcript is barely detectable at E11, but increases gradually through E17. Expression of the 2.1 kb GRR3 mRNA is not detected at E7, but is quite strong by E11. After E11, expression decreases and remains constant from E15-E17.

In situ Hybridization Analysis of the Expression of GDNFR-α, GRR2, and GRR3

In order to provide clues to the potential roles and functional sites of GDNFR-α, GRR2 and GRR3, their expression was examined in regions where biological effects of GDNF have been demonstrated. In the E18 rat embryo, GDNF is highly expressed in the growing ureteric buds and maturing nephrons of the kidney as well as in the enteric neurons of the intestine. GDNFR-α is found in the same regions of the kidney and intestine as GDNF, but is also expressed at moderate levels in both the dorsal and ventral spinal cord. *ret* is expressed in the kidney and intestine as well, although its expression in the kidney seems to be confined to the ureteric buds.

Expression of *ret* is high in the ventral motor neurons, but low in the dorsal region of the spinal cord. Like *ret*, expression of GRR2 in the kidney is restricted to the ureteric buds. GRR2 is expressed in both the dorsal and ventral regions of the spinal cord. A weak, diffuse hybridization signal was detected in the liver for GDNF, *ret*, and GDNFR-α.

In the postnatal day 7 rat, *ret* expression can be detected at substantial levels in the substantia nigra, trigeminal ganglia, and at a lower level in the reticular thalamic nucleus. GDNFR-α expression is high in both the reticular and ventromedial thalamic nuclei as well as in the medial habenular nucleus. Moderate expression of GDNFR-α is observed in the substantia nigra and lower but detectable levels are found in the hippocampus. GRR2 is expressed at moderate levels in the reticular thalamic nucleus, ventromedial thalamic nucleus, cerebral cortex (especially the cingulate cortex), and the substantia nigra. We could detect no expression of GRR3 in the P7 rat brain, but significant expression could be detected in the trigeminal ganglia.

Discussion

This study describes the isolation of GRR3, a novel molecule related to GDNFR-α and GRR2 and compares the tissue expression of *ret* with that of all three members of the GDNFR receptor family. GRR2 is 48% identical in amino acid sequence to GDNFR-α, while GRR3 is somewhat more distantly related at 35% identity. The position of 28 cysteine residues are conserved in all three molecules. Like GDNFR-α, both GRR2 and GRR3 have hydrophobic C-termini that are likely to be involved in GPI linkage to the cell membrane, and neither has a cytoplasmic domain. This strong conservation of sequence and structural features suggests that GDNFR-α, GRR2, and GRR3 define a new family of receptors for GDNF and related ligands. GDNF signaling is initiated by binding to GDNFR-α and accomplished by association and consequent activation of the Ret PTK. Based upon its sequence and structural similarities to GDNFR-α and GRR2, GRR3 is likely to function as a binding partner for GDNF, neurturin, and/or some other as yet undiscovered member of this ligand family.

The expression patterns of GDNFR-α, GRR2, and GRR3 in adult rat tissues are similar but distinct. All three mRNAs are found in lung, spleen, heart, and kidney while none of the three show significant expression in muscle or testis. Adult brain exhibits high expression of GDNFR-α and GRR2 mRNAs, but little or no GRR3 is detected. Expression of GDNFR-α mRNA is high in liver while GRR2 mRNA is almost nonexistent. If GDNF, neurturin and other as yet undiscovered GDNF-like

ligands signal exclusively through Ret, differences in expression patterns of the ligand-specific binding receptors could provide a mechanism for ligand tissue specificity. Since the expression of *c-ret* can be detected throughout the period from E8.5 to E16.5, differences in the temporal expression of the receptor proteins could also define ligand specificity during development.

Expression of all the receptors and of *c-ret* is high in the adult kidney, the site of the most severe defects found in Ret knockout animals. *In situ* hybridization analyses indicate that *ret*, GDNFR-α, GRR2 and GRR3 are colocalized in several tissues, suggesting that GRR2 and GRR3 may also exert their *in vivo* effects through interaction with Ret (Table 5).

Table 5
Expression of *ret*, GDNFR-α, GRR2, and GRR3
in embryonic day 18 rat

	ret	GDNFR-α	GRR2	GRR3
Kidney/Intestine	+++	+++	++	-*
Brain:				
Thalamic Nuclei:				
Reticular	++	+++	++	-
Ventral medial	+	+++	++	-
Substantia Nigra	+++	+++	+++	-
Habenular nucleus	-	+++	-	-
Hippocampus	+/-	++	-	-
Spinal cord:				
Dorsal	+	++	++	-
Ventral	++	+++	++	-
Trigeminal Ganglia	+++	+++	-	+++

* High levels of expression were detected in the adult kidney.

Both GDNFR-α and GRR2 are transcribed along with *ret* in the kidney and intestine, in the substantia nigra, in the thalamus, and in ventral spinal motor neurons.

This finding is consistent with GDNF's ability to promote the survival of dopaminergic and motor neurons and with the phenotypes of the Ret and GDNF knockout animals. Although little expression of GRR3 was found in the brain, it is co-expressed with *ret* and GDNFR-α in the trigeminal ganglia in E18 and P7 rats. These observations indicate that GDNF action may be regulated by association with different binding components depending on the tissue and developmental stage, while always signaling through Ret.

Although expression of *ret* is often co-localized with that of GDNFR-α, GRR2 and GRR3, there are several sites that express one or more of the binding receptors at high levels while *ret* expression is undetectable. Little or no *ret* is expressed in the spleen or lung where all three receptors are expressed at high levels. High levels of GDNFR-α mRNA are found in the liver, medial habenular nucleus, and the hippocampus, and GRR2 expression is prominent in the cortex. Little *ret* expression was observed in either of these regions. The lack of *ret* expression at some sites of substantial GDNFR expression suggests that either a signaling partner other than Ret may be employed by the GDNFRs in these tissues or that the receptors have an alternate mechanism of action. Two possibilities are that the receptors may act to sequester ligands of the GDNF family or that some fraction of the membrane bound receptors are released and mediate ligand function as soluble receptors.

Experimental Procedures

Cloning of GRR3

The GenBank database was searched for sequences related to GDNFR-α and GRR2 using the Wisconsin sequence analysis package (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, WI). Oligonucleotide primers corresponding to regions near the ends of the EST AA238748.Gb_New2 were synthesized. Primers corresponding to AA238748.Gb_New2 were used for PCR screening of 83 pools of 1000 clones each from a rat E15 embryonic cDNA library. A single positive pool was identified by this method. The DNA fragment amplified from this pool was subcloned into a plasmid vector, and the insert was sequenced using an Applied Biosystems 373A automated DNA sequencer with Taq DyeDeoxy Terminator cycle sequencing kits (Applied Biosystems, Foster City, CA). The insert was then labeled with [^{32}P]-dCTP using a Random Primed DNA Labeling Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Clones from the cDNA library pool that had been identified as positive by PCR were plated on 15 cm agarose plates and replicated on duplicate nitrocellulose filters for screening by hybridization to

the radiolabeled insert. Filters were prehybridized at 55°C for 3.5 hours in 200 ml of 6 x SSC, 1 x Denhardts, 0.5% SDS, and 50 µg/ml salmon sperm DNA. Following the addition of 2 x 10^8 cpm of the radiolabeled probe, hybridization was continued for 18 hours. Filters were then washed twice for 30 minutes each at 55°C in 0.2 x SSC, 0.1% SDS and exposed to X-ray film overnight with an intensifying screen.

DNA Sequencing and Sequence Analysis

DNA from clones that screened positively by hybridization was prepared and sequenced using an automated Applied Biosystems 373A DNA sequencer and Taq DyeDeoxy Terminator cycle sequencing kits (Applied Biosystems, Foster City, CA). The peptide sequences of GDNFR-α, GRR2, and GRR3 were aligned using the Lineup program (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, WI).

Blot Hybridization Analysis

For blot hybridization analysis, the cloned rat GRR3 cDNA was labeled using the Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Rat and mouse RNA blots (Clontech) were hybridized with the probe and washed at high stringency using the reagents of the ExpressHyb Kit (Clontech, Palo Alto, CA) according to the instructions of the manufacturer. Following exposure on X-ray film, the filters were stripped of probe by boiling in 0.5% SDS for 10 minutes and rehybridized with a β-actin probe (Clontech, Palo Alto, CA) as a control for total RNA loading.

In situ Hybridization

In situ hybridization using anti-sense riboprobes of GDNF, *ret*, GDNFR-α, GRR2, and GRR3, was done according to Zhou et al. (Journal Of Neuroscience Research, 37, 129-143, 1994). The *ret* probe is a 316 nt fragment derived from the extracellular domain of the rat *ret* cDNA. GDNF mRNA was detected using a 303 nt fragment of a rat GDNF cDNA clone (nucleotide #50 to 352, Lin et al., 1993). GDNFR-α transcripts were detected with a 396 nt riboprobe (nucleotides 1072 to 1468). GRR2 transcripts were detected with a 205 nt antisense riboprobe corresponding to amino acids 339-413 (Figure 26). GRR3 transcripts were detected with a 225 nt antisense riboprobe corresponding to amino acids 239-315 (Figure 26).

While the present invention has been described in terms of preferred

embodiments and exemplary nucleic acid and amino acid sequences, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

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References

Angrist, M., Bolk, S., Thiel, B., Puffenberger, E.G., Hofstra, R.M., Buys, C.H., Cass, D.T., and Chakravarti, A. (1995). Mutation analysis of the RET receptor tyrosine kinase in Hirschsprung disease. Hum. Mol.Genet.4, 821-830.

Arenas, E., Trupp, M., Akerud, P., and Ibanez, C.F. (1995). GDNF Prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. Neuron 15, 1465-1473.

Aruffo, A. and Seed, B. (1987). Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. Proceedings Of The National Academy Of Sciences Of The United States Of America. 84, 8573-8577.

Bartley, T.D., Hunt, R.W., Welcher, A.A., Boyle, W.J., Parker, V.P., Lindberg, R.A., Lu, H.S., Colombero, A.M., Elliott, R.L., Guthrie, B.A., Holst, P.L., Skrine, J.D., Toso, R.J., Zhang, M., Fernandez, E., Trail, G., Varnum, B., Yarden, Y., Hunter, T., and Fox, G.M. (1994). B61 is a Ligand for the ECK Receptor protein-tyrosine kinase. Nature. 368, 558-560.

Beck, K.D., Valverde, J., Alexi, T., Poulsen, K., Moffat, B., Vandlen, R.A., Rosenthal, A., and Hefti, F. (1995). Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. Nature. 373, 339-341.

Camu, W. and Henderson, C. (1992). Purification of embryonic rat motoneurons by panning on a monoclonal antibody to the low-affinity NGF receptor. J Neuroscience. 44, 59-70.

Choi-Lundberg, D.L. and Bohn, M.C. (1995). Ontogeny and distribution of glial cell line-derived neurotrophic factor (GDNF) mRNA in rat. Brain Res. Dev. Brain Res. 85, 80-88.

Davis, S., Aldrich, T.H., Valenzuela, D.M., Wong, V.V., Furth, M.E., Squinto, S.P., and Yancopoulos, G.D. (1991). The receptor for ciliary neurotrophic factor. Science. 253, 59-63.

Donis-Keller, H., Dou, S., Chi, D., Carlson, K., Toshima, K., Lairmore, T., Howe, J., Moley, J., Goodfellow, P. and Wells, S. (1993). Mutations in the ret proto-oncogene are associated with MEN 2A and FMTC. Hum. Molec. Genet. 2, 851-856.

Ebendal, T., Tomac, A., Hoffer, B.J., and Olson, L. (1995). Glial cell line-derived neurotrophic factor stimulates fiber formation and survival in cultured neurons from peripheral autonomic ganglia. Journal Of Neuroscience Research. 40, 276-284.

Economides, A.N., Ravetch, J.V., Yancopoulos, G.D., and Stahl, N. (1995). Designer cytokines: targeting actions to cells of choice. Science 270, 1351-1353.

Edery, P., Lyonnet, S., Mulligan, L., Pelet, A., Dow, E., Abel, L., Holder, S., Nihoul-Fekete, C., Ponder, B. and Munnich, A. (1994). Mutations of the ret proto-oncogene in Hirschsprug's disease. Nature. 367, 378-380.

Gearing, D.P., King, J.A., Gough, N.M., and Nicola, N.A. (1989). Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. EMBO Journal 8, 3667-3676.

Henderson, C.E., Phillips, H.S., Pollock, R.A., Davies, A.M., Lemeulle, C., Armanini, M., Simpson, L.C., Moffet, B., Vandlen, R.A., Koliatsos, V.E., and et al (1994). GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science. 266, 1062-1064.

Hoffer, B.J., Hoffman, A., Bowenkamp, K., Huettl, P., Hudson, J., Martin, D., Lin, L.F., and Gerhardt, G.A. (1994). Glial cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic neurons in vivo. Neuroscience Letters. 182, 107-111.

Hofstra, R., Landsvater, R., Ceccherini, I., Stulp, R., Stelwagen, T., Luo, Y., Pasini, B., Hoppener, J., van Amstel, H., Romeo, G., Lips, C. and Buys, C. (1994). A mutation in the ret proto-oncogene associated with multipleendocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. Nature. 367, 375-376.

Ikeda, I., Ishizaka, Y., Tahira, T., Suzuki, T., Onda, M., Sugimura, T., and Nagao, M. (1990). Specific expression of the ret proto-oncogene in human neuroblastoma

cell lines. Oncogene. 5, 1291-1296.

Ip, N.Y., Nye, S.H., Boulton, T.G., Davis, S., Yasukawa, K., Kishimoto, T., Anderson, D.J., and et al (1992). CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. Cell. 69, 1121-1132.

Iwamoto, T., Taniguchi, M., Asia, N., Ohkusu, K., Nakashima, I. and Takahashi, M. (1993). cDNA cloning of mouse ret proto-oncongene and its sequence similarity to the cadherin superfamily. Oncogene. 8, 1087-1091.

Jing, S.Q., Spencer, T., Miller, K., Hopkins, C., and Trowbridge, I.S. (1990). Role of the human transferrin receptor cytoplasmic domain in endocytosis: localization of a specific signal sequence for internalization. Journal Of Cell Biology. 110, 283-294.

Kearns, C.M. and Gash, D.M. (1995). GDNF protects nigral dopamine neurons against 6-hydroxydopamine in vivo. Brain Research. 672, 104-111.

Kozak, M. (1987). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Research. 15, 8125-8148.

Li, L., Wu, W., Lin, L.F., Lei, M., Oppenheim, R.W., and Houenou, L.J. (1995). Rescue of adult mouse motoneurons from injury-induced cell death by glial cell line-derived neurotrophic factor. Proceedings Of The National Academy Of Sciences Of The United States Of America. 92, 9771-9775.

Lin, L-F.H., Doherty, D.H., Lile, J.D., Bektesh, S., and Collins, F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science. 260, 1130-1132.

Louis, J.C., Magal, E., and Varon, S. (1992). Receptor-mediated toxicity of norepinephrine on cultured catecholaminergic neurons of the rat brain stem. Journal Of Pharmacology And Experimental Therapeutics. 262, 1274-1283.

Mount, H.T., Dean, D.O., Alberch, J., Dreyfus, C.F., and Black, I.B. (1995). Glial cell line-derived neurotrophic factor promotes the survival and morphologic differentiation of Purkinje cells. Proceedings Of The National Academy Of Sciences

Of The United States Of America. 92, 9092-9096.

Mulligan, L., Kwok, J., Healey, C., Elsdon, M., Eng, C., Gardner, E., Love, D., Mole, S., Moore, J., Papi, L., Ponder, M., Telenius, H., Tunnacliffe, A. and Ponder, A. (1993). Germ-line mutations of the ret proto-oncongene in mutiple endocrine neoplasia type 2A. Nature. 363, 458-460.

Oppenheim, R.W., Houenou, L.J., Johnson, J.E., Lin, L.F., Li, L., Lo, A.C., Newsome, A.L., Prevette, D.M., and Wang, S. (1995). Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. Nature. 373, 344-346.

Pachnis, V., Mankoo, B., and Costantini, F. (1993). Expression of the c-ret proto-oncogene during mouse embryogenesis. Development, 119, 1005-1017.

Pearson, W.R. and Lipman, D.J. (1988). Improved tools for biological sequence comparison. Proceedings Of The National Academy Of Sciences Of The United States Of America. 85, 2444-2448.

Poulsen, K.T., Armanini, M.P., Klein, R.D., Hynes, M.A., Phillips, H.S., and Rosenthal, A. (1994). TGF beta 2 and TGF beta 3 are potent survival factors for midbrain dopaminergic neurons. Neuron. 13, 1245-1252.

Romeo, G., Patrizia, R, Luo, Y., Barone, V., Seri, M., Ceccherini, I., Pasini, B., Bocciardi, R., Lerone, M., Kaariainen, H. and Maartucciello, G. (1994). Point mutations affecting the tyrosine kinase domain of the ret proto-oncogene in Hirschsprung's disease. Nature. 367, 377-378.

Santoro, M., Carlomagno, F., Romeo, A., Bottaro, D., Dathan, N., Grieco, M., Fusco, A., Vecchio, G., Matoskova, B., Kraus, M. and Di Fiore, P. (1995). Activation of ret as a dominant transforming gene by germline mutations of MEN2A and MEN2B. Science. 267, 381-383.

Sauer, H., Rosenblad, C., and Bjoerklund, A. (1995). Glial cell line-derived neurotrophic factor but not transforming growth factor beta 3 prevents delayed degeneration of nigral dopaminergic neurons following striatal 6-hydroxydopamine lesion. Proceedings Of The National Academy Of Sciences Of The United States Of

America. 92, 8935-8939.

Saxen, L. (1987). Organogenesis of the kidney. Development and Cell Biology series, Cambridge University Press, Cambridge, England.

Schaar, D.G., Sieber, B.A., Dreyfus, C.F., and Black, I.B. (1993). Regional and cell-specific expression of GDNF in rat brain. Experimental Neurology. 124, 368-371.

Schaar, D.G., Sieber, B.A., Sherwood, A.C., Dean, D., Mendoza, G., Ramakrishnan, L., Dreyfus, C.F., and Black, I.B. (1994). Multiple astrocyte transcripts encode nigral trophic factors in rat and human. Experimental Neurology. 130, 387-393.

Schlessinger, J. and Ullrich, A. (1992). Growth factor signaling by receptor tyrosine kinases. Neuron 9, 383-391.

Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F. and Pachnis, V. (1994). Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor ret. Nature. 367, 380-383.

Segarini, P.R., Ziman, J.M., Kane, C.J., and Dasch, J.R. (1992). Two novel patterns of transforming growth factor beta (TGF-beta) binding to cell surface proteins are dependent upon the binding of TGF-beta 1 and indicate a mechanism of positive cooperativity. Journal Of Biological Chemistry. 267, 1048-1053.

Springer, J.E., Mu, X., Bergmann, L.W., and Trojanowski, J.Q. (1994). Expression of GDNF mRNA in rat and human nervous tissue. Experimental Neurology. 127, 167-170.

Stroemberg, I., Bjoerklund, L., Johansson, M., Tomac, A., Collins, F., Olson, L., Hoffer, B., and Humpel, C. (1993). Glial cell line-derived neurotrophic factor is expressed in the developing but not adult striatum and stimulates developing dopamine neurons in vivo. Experimental Neurology. 124, 401-412.

Takahashi, M., Ritz, J. and Cooper, G. (1985). Activation of a novel human tranforming gene, ret, by DNA rearrangement. Cell. 42, 581-588.

Takahashi, M. and Cooper, G. (1987). Ret trasnforming gene encodes a fusion protein homologous to tyrosine kinases. Mol. Cell. Biol., 7, 1378-1385.

Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988). SRa promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. Mol. Cell. Biol. 8, 466-472.

Tomac, A., Lindqvist, E., Lin, L.F., Ogren, S.O., Young, D., Hoffer, B.J., and Olson, L. (1995a). Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. Nature. 373, 335-339.

Tomac, A., Widenfalk, J., Lin, L.F., Kohno, T., Ebendal, T., Hoffer, B.J., and Olson, L. (1995b). Retrograde axonal transport of glial cell line-derived neurotrophic factor in the adult nigrostriatal system suggests a trophic role in the adult. Proceedings Of The National Academy Of Sciences Of The United States Of America. 92, 8274-8278.

Trupp, M., Ryden, M., Joernvall, H., Funakoshi, H., Timmusk, T., Arenas, E., and Ibanez, C.F. (1995). Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. Journal Of Cell Biology. 130, 137-148.

Tsuzuki, T., Takahashi, M., Asai, N., Iwashita, T., Matsuyama, M. and Asai, J. (1995). Spatial and temporal expression of the ret proto-oncongene product in embryonic, infant and adult rat tissues. Oncogene, 10, 191-198.

Ullrich, A and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. Cell, 61, 203-211.

van der Geer, P., Hunter, T., and Lindberg, R.A. (1994). Receptor protein-tyrosine kinases and their signal transduction pathways. 10, 251-337.

van Heyningen, V. (1994). One gene-four syndromes. Nature, 367, 319-320.

von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. Nucleic Acids Research. 14, 4683-4690.

Yan, Q., Matheson, C., and Lopez, O.T. (1995). In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons. Nature. 373, 341-344.

Zurn, A.D., Baetge, E.E., Hammang, J.P., Tan, S.A., and Aebischer, P. (1994). Glial cell line-derived neurotrophic factor (GDNF), a new neurotrophic factor for motoneurones. Neuroreport. 6, 113-118.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Fox, Gary M
Jing, Shuqian
Wen, Duanzhi

(ii) TITLE OF INVENTION: NEUROTROPHIC FACTOR RECEPTORS

(iii) NUMBER OF SEQUENCES: __

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: AMGEN INC
(B) STREET: 1840 DeHavilland Drive
(C) CITY: Thousand Oaks
(D) STATE: CA
(E) COUNTRY: US
(F) ZIP: 91320-1789

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US unknown
(B) FILING DATE: _____
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/017,221
(B) FILING DATE: 09-MAY-1996

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/015,907
(B) FILING DATE: 22-APR-1996

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US _____
(B) FILING DATE: 14-APR-1997
(C) REFERENCE/DOCKET NUMBER: A-401-A

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Curry, Daniel R.
(B) REGISTRATION NUMBER: 32,727
(C) REFERENCE/DOCKET NUMBER: A-401-B

Sub a'

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2568 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 540..1934

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CGGACCTGAA CCCCTAAAAG CGGAACCGCC TCCCGCCCTC GCCATCCCGG AGCTGAGTCG     360

CCGGCGGCGG TGGCTGCTGC CAGACCCGGA GTTTCCTCTT TCACTGGATG GAGCTGAACT     420

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ATG TTC CTG GCG ACC CTG TAC TTC GCG CTG CCG CTC TTG GAC TTG CTC         587
Met Phe Leu Ala Thr Leu Tyr Phe Ala Leu Pro Leu Leu Asp Leu Leu
  1               5                  10                  15

CTG TCG GCC GAA GTG AGC GGC GGA GAC CGC CTG GAT TGC GTG AAA GCC         635
Leu Ser Ala Glu Val Ser Gly Gly Asp Arg Leu Asp Cys Val Lys Ala
             20                  25                  30

AGT GAT CAG TGC CTG AAG GAG CAG AGC TGC AGC ACC AAG TAC CGC ACG         683
Ser Asp Gln Cys Leu Lys Glu Gln Ser Cys Ser Thr Lys Tyr Arg Thr
         35                  40                  45

CTA AGG CAG TGC GTG GCG GGC AAG GAG ACC AAC TTC AGC CTG GCA TCC         731
Leu Arg Gln Cys Val Ala Gly Lys Glu Thr Asn Phe Ser Leu Ala Ser
     50                  55                  60

GGC CTG GAG GCC AAG GAT GAG TGC CGC AGC GCC ATG GAG GCC CTG AAG         779
Gly Leu Glu Ala Lys Asp Glu Cys Arg Ser Ala Met Glu Ala Leu Lys
 65                  70                  75                  80
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85 90 95

AAG AAC TGC CTG CGC ATT TAC TGG AGC ATG TAC CAG AGC CTG CAG GGA 875
Lys Asn Cys Leu Arg Ile Tyr Trp Ser Met Tyr Gln Ser Leu Gln Gly
100 105 110

AAT GAT CTG CTG GAG GAT TCC CCA TAT GAA CCA GTT AAC AGC AGA TTG 923
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115 120 125

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130 135 140

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145 150 155 160

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165 170 175

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180 185 190

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275 280 285

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290 295 300

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305                 310                 315                 320

GAC CTA GAA GAG TGC TTG AAA TTT TTG AAT TTC TTC AAG GAC AAT ACA        1547
Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn Thr
                325                 330                 335

TGT CTT AAA AAT GCA ATT CAA GCC TTT GGC AAT GGC TCC GAT GTG ACC        1595
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            340                 345                 350

GTG TGG CAG CCA GCC TTC CCA GTA CAG ACC ACC ACT GCC ACT ACC ACC        1643
Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr Thr Ala Thr Thr Thr
        355                 360                 365

ACT GCC CTC CGG GTT AAG AAC AAG CCC CTG GGG CCA GCA GGG TCT GAG        1691
Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser Glu
    370                 375                 380

AAT GAA ATT CCC ACT CAT GTT TTG CCA CCG TGT GCA AAT TTA CAG GCA        1739
Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln Ala
385                 390                 395                 400

CAG AAG CTG AAA TCC AAT GTG TCG GGC AAT ACA CAC CTC TGT ATT TCC        1787
Gln Lys Leu Lys Ser Asn Val Ser Gly Asn Thr His Leu Cys Ile Ser
                405                 410                 415

AAT GGT AAT TAT GAA AAA GAA GGT CTC GGT GCT TCC AGC CAC ATA ACC        1835
Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser His Ile Thr
            420                 425                 430

ACA AAA TCA ATG GCT GCT CCT CCA AGC TGT GGT CTG AGC CCA CTG CTG        1883
Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro Leu Leu
        435                 440                 445

GTC CTG GTG GTA ACC GCT CTG TCC ACC CTA TTA TCT TTA ACA GAA ACA        1931
Val Leu Val Val Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr Glu Thr
    450                 455                 460

TCA TAGCTGCATT AAAAAAATAC AATATGGACA TGTAAAAAGA CAAAAACCAA             1984
Ser
465

GTTATCTGTT TCCTGTTCTC TTGTATAGCT GAAATTCCAG TTTAGGAGCT CAGTTGAGAA      2044

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 465 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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            20                  25                  30

Ser Asp Gln Cys Leu Lys Glu Gln Ser Cys Ser Thr Lys Tyr Arg Thr
        35                  40                  45

Leu Arg Gln Cys Val Ala Gly Lys Glu Thr Asn Phe Ser Leu Ala Ser
    50                  55                  60

Gly Leu Glu Ala Lys Asp Glu Cys Arg Ser Ala Met Glu Ala Leu Lys
65                  70                  75                  80

Gln Lys Ser Leu Tyr Asn Cys Arg Cys Lys Arg Gly Met Lys Lys Glu
                85                  90                  95

Lys Asn Cys Leu Arg Ile Tyr Trp Ser Met Tyr Gln Ser Leu Gln Gly
            100                 105                 110

Asn Asp Leu Leu Glu Asp Ser Pro Tyr Glu Pro Val Asn Ser Arg Leu
        115                 120                 125

Ser Asp Ile Phe Arg Val Val Pro Phe Ile Ser Asp Val Phe Gln Gln
    130                 135                 140

Val Glu His Ile Pro Lys Gly Asn Asn Cys Leu Asp Ala Ala Lys Ala
145                 150                 155                 160

Cys Asn Leu Asp Asp Ile Cys Lys Lys Tyr Arg Ser Ala Tyr Ile Thr
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Pro Cys Thr Thr Ser Val Ser Asn Asp Val Cys Asn Arg Arg Lys Cys
            180                 185                 190
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His Lys Ala Leu Arg Gln Phe Phe Asp Lys Val Pro Ala Lys His Ser
        195                 200                 205

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    210                 215                 220

Arg Arg Gln Thr Ile Val Pro Val Cys Ser Tyr Glu Glu Arg Glu Lys
225                 230                 235                 240

Pro Asn Cys Leu Asn Leu Gln Asp Ser Cys Lys Thr Asn Tyr Ile Cys
                245                 250                 255

Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys Gln Pro Glu Ser Arg
            260                 265                 270

Ser Val Ser Ser Cys Leu Lys Glu Asn Tyr Ala Asp Cys Leu Leu Ala
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Tyr Ser Gly Leu Ile Gly Thr Val Met Thr Pro Asn Tyr Ile Asp Ser
    290                 295                 300

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305                 310                 315                 320

Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn Thr
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Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp Val Thr
            340                 345                 350

Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr Thr Ala Thr Thr Thr
        355                 360                 365

Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser Glu
    370                 375                 380

Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln Ala
385                 390                 395                 400

Gln Lys Leu Lys Ser Asn Val Ser Gly Asn Thr His Leu Cys Ile Ser
                405                 410                 415

Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser His Ile Thr
            420                 425                 430

Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro Leu Leu
        435                 440                 445

Val Leu Val Val Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr Glu Thr
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Ser
465
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2138 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 302..1705

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GAACTTTGAG TGGCCAGAGG AGCGCAGTCG CCCGGGGATC GCTGCACGCT GAGCTCTCTC     240

CCCGAGACCG GGCGGCGGCT TTGGATTTTG GGGGGGCGGG GACCAGCTGC GCGGCGGCAC     300

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  Met Phe Leu Ala Thr Leu Tyr Phe Ala Leu Pro Leu Leu Asp Leu
    1               5                  10                  15

CTG ATG TCC GCC GAG GTG AGT GGT GGA GAC CGT CTG GAC TGT GTG AAA       394
Leu Met Ser Ala Glu Val Ser Gly Gly Asp Arg Leu Asp Cys Val Lys
                  20                  25                  30

GCC AGC GAT CAG TGC CTG AAG GAA CAG AGC TGC AGC ACC AAG TAC CGC       442
Ala Ser Asp Gln Cys Leu Lys Glu Gln Ser Cys Ser Thr Lys Tyr Arg
             35                  40                  45

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Thr Leu Arg Gln Cys Val Ala Gly Lys Glu Thr Asn Phe Ser Leu Thr
         50                  55                  60

TCC GGC CTT GAG GCC AAG GAT GAG TGC CGT AGC GCC ATG GAG GCC TTG       538
Ser Gly Leu Glu Ala Lys Asp Glu Cys Arg Ser Ala Met Glu Ala Leu
     65                  70                  75

AAG CAG AAG TCT CTG TAC AAC TGC CGC TGC AAG CGG GGC ATG AAG AAA       586
Lys Gln Lys Ser Leu Tyr Asn Cys Arg Cys Lys Arg Gly Met Lys Lys
 80                  85                  90                  95

GAG AAG AAT TGT CTG CGT ATC TAC TGG AGC ATG TAC CAG AGC CTG CAG       634
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            115                 120                 125

TTG TCA GAT ATA TTC CGG GCA GTC CCG TTC ATA TCA GAT GTT TTC CAG         730
Leu Ser Asp Ile Phe Arg Ala Val Pro Phe Ile Ser Asp Val Phe Gln
        130                 135                 140

CAA GTG GAA CAC ATT TCC AAA GGG AAC AAC TGC CTG GAC GCA GCC AAG         778
Gln Val Glu His Ile Ser Lys Gly Asn Asn Cys Leu Asp Ala Ala Lys
    145                 150                 155

GCC TGC AAC CTG GAC GAC ACC TGT AAG AAG TAC AGG TCG GCC TAC ATC         826
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160                 165                 170                 175

ACC CCC TGC ACC ACC AGC ATG TCC AAC GAG GTC TGC AAC CGC CGT AAG         874
Thr Pro Cys Thr Thr Ser Met Ser Asn Glu Val Cys Asn Arg Arg Lys
                180                 185                 190

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Cys His Lys Ala Leu Arg Gln Phe Phe Asp Lys Val Pro Ala Lys His
            195                 200                 205

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Ser Tyr Gly Met Leu Phe Cys Ser Cys Arg Asp Ile Ala Cys Thr Glu
        210                 215                 220

CGG CGG CGA CAG ACT ATC GTC CCC GTG TGC TCC TAT GAA GAA CGA GAG        1018
Arg Arg Arg Gln Thr Ile Val Pro Val Cys Ser Tyr Glu Glu Arg Glu
    225                 230                 235

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Arg Pro Asn Cys Leu Ser Leu Gln Asp Ser Cys Lys Thr Asn Tyr Ile
240                 245                 250                 255

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Cys Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys Gln Pro Glu Ser
                260                 265                 270

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Arg Ser Val Ser Asn Cys Leu Lys Glu Asn Tyr Ala Asp Cys Leu Leu
            275                 280                 285

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Ala Tyr Ser Gly Leu Ile Gly Thr Val Met Thr Pro Asn Tyr Val Asp
        290                 295                 300

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Ser Ser Ser Leu Ser Val Ala Pro Trp Cys Asp Cys Ser Asn Ser Gly
    305                 310                 315

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Asn Asp Leu Glu Asp Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn
320                 325                 330                 335
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Thr Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp Val
340 345 350

ACC ATG TGG CAG CCA GCC CCT CCA GTC CAG ACC ACC ACT GCC ACC ACT 1402
Thr Met Trp Gln Pro Ala Pro Pro Val Gln Thr Thr Thr Ala Thr Thr
355 360 365

ACC ACT GCC TTC CGG GTC AAG AAC AAG CCT CTG GGG CCA GCA GGG TCT 1450
Thr Thr Ala Phe Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser
370 375 380

GAG AAT GAG ATC CCC ACA CAC GTT TTA CCA CCC TGT GCG AAT TTG CAG 1498
Glu Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln
385 390 395

GCT CAG AAG CTG AAA TCC AAT GTG TCG GGT AGC ACA CAC CTC TGT CTT 1546
Ala Gln Lys Leu Lys Ser Asn Val Ser Gly Ser Thr His Leu Cys Leu
400 405 410 415

TCT GAT AGT GAT TTC GGA AAG GAT GGT CTC GCT GGT GCC TCC AGC CAC 1594
Ser Asp Ser Asp Phe Gly Lys Asp Gly Leu Ala Gly Ala Ser Ser His
420 425 430

ATA ACC ACA AAA TCA ATG GCT GCT CCT CCC AGC TGC AGT CTG AGC TCA 1642
Ile Thr Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Ser Leu Ser Ser
435 440 445

CTG CCG GTG CTG ATG CTC ACC GCC CTT GCT GCC CTG TTA TCT GTA TCG 1690
Leu Pro Val Leu Met Leu Thr Ala Leu Ala Ala Leu Leu Ser Val Ser
450 455 460

TTG GCA GAA ACG TCG TAGCTGCATC CGGGAAAACA GTATGAAAAG ACAAAAGAGA 1745
Leu Ala Glu Thr Ser
465

ACCAAGTATT CTGTCCCTGT CCTCTTGTAT ATCTGAAAAT CCAGTTTTAA AAGCTCCGTT 1805

GAGAAGCAGT TTCACCCAAC TGGAACTCTT TCCTTGTTTT TAAGAAAGCT TGTGGCCCTC 1865

AGGGGCTTCT GTTGAAGAAC TGCTACAGGG CTAATTCCAA ACCCATAAGG CTCTGGGGCG 1925

TGGTGCGGCT TAAGGGGACC ATTTGCACCA TGTAAAGCAA GCTGGGCTTA TCATGTGTTT 1985

GATGGTGAGG ATGGTAGTGG TGATGATGAT GGTAATTTTA ACAGCTTGAA CCCTGTTCTC 2045

TCTACTGGTT AGGAACAGGA GATACTATTG ATAAAGATTC TTCCATGTCT TACTCAGCAG 2105

CATTGCCTTC TGAAGACAGG CCCGCAGCCG TCG 2138

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 468 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Phe Leu Ala Thr Leu Tyr Phe Ala Leu Pro Leu Leu Asp Leu Leu
  1               5                  10                  15

Met Ser Ala Glu Val Ser Gly Gly Asp Arg Leu Asp Cys Val Lys Ala
             20                  25                  30

Ser Asp Gln Cys Leu Lys Glu Gln Ser Cys Ser Thr Lys Tyr Arg Thr
         35                  40                  45

Leu Arg Gln Cys Val Ala Gly Lys Glu Thr Asn Phe Ser Leu Thr Ser
     50                  55                  60

Gly Leu Glu Ala Lys Asp Glu Cys Arg Ser Ala Met Glu Ala Leu Lys
 65                  70                  75                  80

Gln Lys Ser Leu Tyr Asn Cys Arg Cys Lys Arg Gly Met Lys Lys Glu
                 85                  90                  95

Lys Asn Cys Leu Arg Ile Tyr Trp Ser Met Tyr Gln Ser Leu Gln Gly
            100                 105                 110

Asn Asp Leu Leu Glu Asp Ser Pro Tyr Glu Pro Val Asn Ser Arg Leu
        115                 120                 125

Ser Asp Ile Phe Arg Ala Val Pro Phe Ile Ser Asp Val Phe Gln Gln
    130                 135                 140

Val Glu His Ile Ser Lys Gly Asn Asn Cys Leu Asp Ala Ala Lys Ala
145                 150                 155                 160

Cys Asn Leu Asp Asp Thr Cys Lys Lys Tyr Arg Ser Ala Tyr Ile Thr
                165                 170                 175

Pro Cys Thr Thr Ser Met Ser Asn Glu Val Cys Asn Arg Arg Lys Cys
            180                 185                 190

His Lys Ala Leu Arg Gln Phe Phe Asp Lys Val Pro Ala Lys His Ser
        195                 200                 205

Tyr Gly Met Leu Phe Cys Ser Cys Arg Asp Ile Ala Cys Thr Glu Arg
    210                 215                 220

Arg Arg Gln Thr Ile Val Pro Val Cys Ser Tyr Glu Glu Arg Glu Arg
225                 230                 235                 240

Pro Asn Cys Leu Ser Leu Gln Asp Ser Cys Lys Thr Asn Tyr Ile Cys
                245                 250                 255

Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys Gln Pro Glu Ser Arg
            260                 265                 270
```

```
Ser Val Ser Asn Cys Leu Lys Glu Asn Tyr Ala Asp Cys Leu Leu Ala
        275                 280                 285

Tyr Ser Gly Leu Ile Gly Thr Val Met Thr Pro Asn Tyr Val Asp Ser
    290                 295                 300

Ser Ser Leu Ser Val Ala Pro Trp Cys Asp Cys Ser Asn Ser Gly Asn
305                 310                 315                 320

Asp Leu Glu Asp Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn Thr
                325                 330                 335

Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp Val Thr
            340                 345                 350

Met Trp Gln Pro Ala Pro Pro Val Gln Thr Thr Thr Ala Thr Thr Thr
        355                 360                 365

Thr Ala Phe Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser Glu
    370                 375                 380

Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln Ala
385                 390                 395                 400

Gln Lys Leu Lys Ser Asn Val Ser Gly Ser Thr His Leu Cys Leu Ser
                405                 410                 415

Asp Ser Asp Phe Gly Lys Asp Gly Leu Ala Gly Ala Ser Ser His Ile
            420                 425                 430

Thr Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Ser Leu Ser Ser Leu
        435                 440                 445

Pro Val Leu Met Leu Thr Ala Leu Ala Ala Leu Leu Ser Val Ser Leu
    450                 455                 460

Ala Glu Thr Ser
465
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 3209 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..539
- (D) OTHER INFORMATION: /note= "1 to 539 is -237 to 301 of Figure 5 Gdnfr"

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 540..1937

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
AATCTGGCCT CGGAACACGC CATTCTCCGC GCCGCTTCCA ATAACCACTA ACATCCCTAA      60

CGAGCATCCG AGCCGAGGGC TCTGCTCGGA AATCGTCCTG GCCCAACTCG GCCCTTCGAG     120

CTCTCGAAGA TTACCGCATC TATTTTTTTT TTCTTTTTTT TCTTTTCCTA GCGCAGATAA     180

AGTGAGCCCG GAAAGGGAAG GAGGGGGCGG GGACACCATT GCCCTGAAAG AATAAATAAG     240

TAAATAAACA AACTGGCTCC TCGCCGCAGC TGGACGCGGT CGGTTGAGTC CAGGTTGGGT     300

CGGACCTGAA CCCCTAAAAG CGGAACCGCC TCCCGCCCTC GCCATCCCGG AGCTGAGTCG     360

CCGGCGGCGG TGGCTGCTGC CAGACCCGGA GTTTCCTCTT TCACTGGATG GAGCTGAACT     420

TTGGGCGGCC AGAGCAGCAC AGCTGTCCGG GGATCGCTGC ACGCTGAGCT CCCTCGGCAA     480

GACCCAGCGG CGGCTCGGGA TTTTTTTGGG GGGGCGGGGA CCAGCCCCGC GCCGGCACC      539

ATG TTC CTG GCG ACC CTG TAC TTC GCG CTG CCG CTC TTG GAC TTG CTC        587
Met Phe Leu Ala Thr Leu Tyr Phe Ala Leu Pro Leu Leu Asp Leu Leu
  1               5                  10                  15

CTG TCG GCC GAA GTG AGC GGC GGA GAC CGC CTG GAT TGC GTG AAA GCC        635
Leu Ser Ala Glu Val Ser Gly Gly Asp Arg Leu Asp Cys Val Lys Ala
             20                  25                  30

AGT GAT CAG TGC CTG AAG GAG CAG AGC TGC AGC ACC AAG TAC CGC ACG        683
Ser Asp Gln Cys Leu Lys Glu Gln Ser Cys Ser Thr Lys Tyr Arg Thr
         35                  40                  45

CTA AGG CAG TGC GTG GCG GGC AAG GAG ACC AAC TTC AGC CTG GCA TCC        731
Leu Arg Gln Cys Val Ala Gly Lys Glu Thr Asn Phe Ser Leu Ala Ser
     50                  55                  60

GGC CTG GAG GCC AAG GAT GAG TGC CGC AGC GCC ATG GAG GCC CTG AAG        779
Gly Leu Glu Ala Lys Asp Glu Cys Arg Ser Ala Met Glu Ala Leu Lys
 65                  70                  75                  80

CAG AAG TCG CTC TAC AAC TGC CGC TGC AAG CGG GGT ATG AAG AAG GAG        827
Gln Lys Ser Leu Tyr Asn Cys Arg Cys Lys Arg Gly Met Lys Lys Glu
                 85                  90                  95

AAG AAC TGC CTG CGC ATT TAC TGG AGC ATG TAC CAG AGC CTG CAG GGA        875
Lys Asn Cys Leu Arg Ile Tyr Trp Ser Met Tyr Gln Ser Leu Gln Gly
            100                 105                 110

AAT GAT CTG CTG GAG GAT TCC CCA TAT GAA CCA GTT AAC AGC AGA TTG        923
Asn Asp Leu Leu Glu Asp Ser Pro Tyr Glu Pro Val Asn Ser Arg Leu
        115                 120                 125
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TCA GAT ATA TTC CGG GTG GTC CCA TTC ATA TCA GAT GTT TTT CAG CAA      971
Ser Asp Ile Phe Arg Val Val Pro Phe Ile Ser Asp Val Phe Gln Gln
    130                 135                 140

GTG GAG CAC ATT CCC AAA GGG AAC AAC TGC CTG GAT GCA GCG AAG GCC     1019
Val Glu His Ile Pro Lys Gly Asn Asn Cys Leu Asp Ala Ala Lys Ala
145                 150                 155                 160

TGC AAC CTC GAC GAC ATT TGC AAG AAG TAC AGG TCG GCG TAC ATC ACC     1067
Cys Asn Leu Asp Asp Ile Cys Lys Lys Tyr Arg Ser Ala Tyr Ile Thr
                165                 170                 175

CCG TGC ACC ACC AGC GTG TCC AAN GAT GTC TGC AAC CGC CGC AAG TGC     1115
Pro Cys Thr Thr Ser Val Ser Xaa Asp Val Cys Asn Arg Arg Lys Cys
            180                 185                 190

CAC AAG GCC CTC CGG CAG TTC TTT GAC AAG GTC CCG GCC AAG CAC AGC     1163
His Lys Ala Leu Arg Gln Phe Phe Asp Lys Val Pro Ala Lys His Ser
        195                 200                 205

TAC GGA ATG CTC TTC TGC TCC TGC CGG GAC ATC GCC TGC ACA GAG CGG     1211
Tyr Gly Met Leu Phe Cys Ser Cys Arg Asp Ile Ala Cys Thr Glu Arg
    210                 215                 220

AGG CGA CAG ACC ATC GTG CCT GTG TGC TCC TAT GAA GAG AGG GAG AAG     1259
Arg Arg Gln Thr Ile Val Pro Val Cys Ser Tyr Glu Glu Arg Glu Lys
225                 230                 235                 240

CCC AAC TGT TTG AAT TTG CAG GAC TCC TGC AAG ACG AAT TAC ATC TGC     1307
Pro Asn Cys Leu Asn Leu Gln Asp Ser Cys Lys Thr Asn Tyr Ile Cys
                245                 250                 255

AGA TCT CGC CTT GCG GAT TTT TTT ACC AAC TGC CAG CCA GAG TCA AGG     1355
Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys Gln Pro Glu Ser Arg
            260                 265                 270

TCT GTC AGC AGC TGT CTA AAG GAA AAC TAC GCT GAC TGC CTC CTC GCC     1403
Ser Val Ser Ser Cys Leu Lys Glu Asn Tyr Ala Asp Cys Leu Leu Ala
        275                 280                 285

TAC TCG GGG CTT ATT GGC ACA GTC ATG ACC CCC AAC TAC ATA GAC TCC     1451
Tyr Ser Gly Leu Ile Gly Thr Val Met Thr Pro Asn Tyr Ile Asp Ser
    290                 295                 300

AGT AGC CTC AGT GTG GCC CCA TGG TGT GAC TGC AGC AAC AGT GGG AAC     1499
Ser Ser Leu Ser Val Ala Pro Trp Cys Asp Cys Ser Asn Ser Gly Asn
305                 310                 315                 320

GAC CTA GAA GAG TGC TTG AAA TTT TTG AAT TTC TTC AAG GAC AAT ACA     1547
Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn Thr
                325                 330                 335

TGT CTT AAA AAT GCA ATT CAA GCC TTT GGC AAT GGC TCC GAT GTG ACC     1595
Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp Val Thr
            340                 345                 350
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GTG TGG CAG CCA GCC TTC CCA GTA CAG ACC ACC ACT GCC ACT ACC ACC      1643
Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr Thr Ala Thr Thr Thr
        355                 360                 365

ACT GCC CTC CGG GTT AAG AAC AAG CCC CTG GGG CCA GCA GGG TCT GAG      1691
Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser Glu
    370                 375                 380

AAT GAA ATT CCC ACT CAT GTT TTG CCA CCG TGT GCA AAT TTA CAG GCA      1739
Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln Ala
385                 390                 395                 400

CAG AAG CTG AAA TCC AAT GTG TCG GGC AAT ACA CAC CTC TGT ATT TCC      1787
Gln Lys Leu Lys Ser Asn Val Ser Gly Asn Thr His Leu Cys Ile Ser
                405                 410                 415

AAT GGT AAT TAT GAA AAA GAA GGT CTC GGT GCT TCC AGC CAC ATA ACC      1835
Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser His Ile Thr
            420                 425                 430

ACA AAA TCA ATG GCT GCT CCT CCA AGC TGT GGT CTG AGC CCA CTG CTG      1883
Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro Leu Leu
        435                 440                 445

GTC CTG GTG GTA ACC GCT CTG TCC ACC CTA TTA TCT TTA ACA GAA ACA      1931
Val Leu Val Val Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr Glu Thr
    450                 455                 460

TCA TAG CTGCATTAAA AAAATACAAT ATGGACATGT AAAAAGACAA AAACCAAGTT      1987
Ser  *
465

ATCTGTTTCC TGTTCTCTTG TATAGCTGAA ATTCCAGTTT AGGAGCTCAG TTGAGAAACA    2047

GTTCCATTCA ACTGGAACAT TTTTTTTTTT NCCTTTTAAG AAAGCTTCTT GTGATCCTTC    2107

GGGGCTTCTG TGAAAAACCT GATGCAGTGC TCCATCCAAA CTCAGAAGGC TTTGGGATAT    2167

GCTGTATTTT AAAGGGACAG TTTGTAACTT GGGCTGTAAA GCAAACTGGG GCTGTGTTTT    2227

CGATGATGAT GATCATCATG ATCATGATNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN    2287

NNNNNNNGAT TTTAACAGTT TTACTTCTGG CCTTTCCTAG CTAGAGAAGG AGTTAATATT    2347

TCTAAGGTAA CTCCCATATC TCCTTTAATG ACATTGATTT CTAATGATAT AAATTTCAGC    2407

CTACATTGAT GCCAAGCTTT TTTGCCACAA AGAAGATTCT TACCAAGAGT GGGCTTTGTG    2467

GAAACAGCTG GTACTGATGT TCACCTTTAT ATATGTACTA GCATTTTCCA CGCTGATGTT    2527

TATGTACTGT AAACAGTTCT GCACTCTTGT ACAAAAGAAA AAACACCTGT CACATCCAAA    2587

TATAGTATCT GTCTTTTCGT CAAAATAGAG AGTGGGGAAT GAGTGTGCCG ATTCAATACC    2647

TCAATCCCTG AACGACACTC TCCTAATCCT AAGCCTTACC TGAGTGAGAA GCCCTTTACC    2707
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TAACAAAAGT CCAATATAGC TGAAATGTCG CTCTAATACT CTTTACACAT ATGAGGTTAT    2767

ATGTAGAAAA AAATTTTACT ACTAAATGAT TTCAACTATT GGCTTTCTAT ATTTTGAAAG    2827

TAATGATATT GTCTCATTTT TTTACTGATG GTTTAATACA AAATACACAG AGCTTGTTTC    2887

CCCTCATAAG TAGTGTTCGC TCTGATATGA ACTTCACAAA TACAGCTCAT CAAAAGCAGA    2947

CTCTGAGAAG CCTCGTGCTG TAGCAGAAAG TTCTGCATCA TGTGACTGTG GACAGGCAGG    3007

AGGAAACAGA ACAGACAAGC ATTGTCTTTT GTCATTGCTC GAAGTGCAAG CGTGCATACC    3067

TGTGGAGGGA ACTGGTGGCT GCTTGTAAAT GTTCTGCAGC ATCTCTTGAC ACACTTGTCA    3127

TGACACAATC CAGTACCTTG GTTTTCAGGT TATCTGACAA AGGCAGCTTT GATTGGGACA    3187

TGGAGGCATG GGCAGGCCGG AA                                             3209
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 466 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Phe Leu Ala Thr Leu Tyr Phe Ala Leu Pro Leu Leu Asp Leu Leu
 1               5                  10                  15

Leu Ser Ala Glu Val Ser Gly Gly Asp Arg Leu Asp Cys Val Lys Ala
            20                  25                  30

Ser Asp Gln Cys Leu Lys Glu Gln Ser Cys Ser Thr Lys Tyr Arg Thr
        35                  40                  45

Leu Arg Gln Cys Val Ala Gly Lys Glu Thr Asn Phe Ser Leu Ala Ser
    50                  55                  60

Gly Leu Glu Ala Lys Asp Glu Cys Arg Ser Ala Met Glu Ala Leu Lys
65                  70                  75                  80

Gln Lys Ser Leu Tyr Asn Cys Arg Cys Lys Arg Gly Met Lys Lys Glu
                85                  90                  95

Lys Asn Cys Leu Arg Ile Tyr Trp Ser Met Tyr Gln Ser Leu Gln Gly
            100                 105                 110

Asn Asp Leu Leu Glu Asp Ser Pro Tyr Glu Pro Val Asn Ser Arg Leu
        115                 120                 125

Ser Asp Ile Phe Arg Val Val Pro Phe Ile Ser Asp Val Phe Gln Gln
    130                 135                 140
```

Val Glu His Ile Pro Lys Gly Asn Asn Cys Leu Asp Ala Ala Lys Ala
145 150 155 160

Cys Asn Leu Asp Asp Ile Cys Lys Lys Tyr Arg Ser Ala Tyr Ile Thr
165 170 175

Pro Cys Thr Thr Ser Val Ser Xaa Asp Val Cys Asn Arg Arg Lys Cys
180 185 190

His Lys Ala Leu Arg Gln Phe Phe Asp Lys Val Pro Ala Lys His Ser
195 200 205

Tyr Gly Met Leu Phe Cys Ser Cys Arg Asp Ile Ala Cys Thr Glu Arg
210 215 220

Arg Arg Gln Thr Ile Val Pro Val Cys Ser Tyr Glu Glu Arg Glu Lys
225 230 235 240

Pro Asn Cys Leu Asn Leu Gln Asp Ser Cys Lys Thr Asn Tyr Ile Cys
245 250 255

Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys Gln Pro Glu Ser Arg
260 265 270

Ser Val Ser Ser Cys Leu Lys Glu Asn Tyr Ala Asp Cys Leu Leu Ala
275 280 285

Tyr Ser Gly Leu Ile Gly Thr Val Met Thr Pro Asn Tyr Ile Asp Ser
290 295 300

Ser Ser Leu Ser Val Ala Pro Trp Cys Asp Cys Ser Asn Ser Gly Asn
305 310 315 320

Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn Thr
325 330 335

Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp Val Thr
340 345 350

Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr Thr Ala Thr Thr Thr
355 360 365

Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser Glu
370 375 380

Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln Ala
385 390 395 400

Gln Lys Leu Lys Ser Asn Val Ser Gly Asn Thr His Leu Cys Ile Ser
405 410 415

Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser His Ile Thr
420 425 430

Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro Leu Leu
        435   440   445

Val Leu Val Val Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr Glu Thr
    450   455   460

Ser *
465

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 508 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..508
(D) OTHER INFORMATION: /note= "1 to 508 is -237 to 272 of Figure 5 Hsgr-21af"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCTGGCCTCG GAACACGCCA TTCTCCGCGC CGCTTCCAAT AACCACTAAC ATCCCTAACG 60

AGCATCCGAG CCGAGGGCTC TGCTCGGAAA TCGTCCTGGC CCAACTCGGC CCTTCGAGCT 120

CTCGAAGATT ACCGCATCTA TTTTTTTTTT CTTTTTTTTC TTTTCCTAGC GCAGATAAAG 180

TGAGCCCGGA AAGGGAAGGA GGGGGCGGGG ACACCATTGC CCTGAAAGAA TAAATAAGTA 240

AATAAACAAA CTGGCTCCTC GCCGCAGCTG GACGCGGTCG GTTGAGTCCA GGTTGGGTCG 300

GACCTGAACC CCTAAAAGCG GAACCGCCTC CCGCCCTCGC CATCCCGGAG CTGAGTCGCC 360

GGCGGCGGTG GCTGCTGCCA GACCCGGAGT TTCCTCTTTC ACTGGATGGA GCTGAACTTT 420

GGGCGGCCAG AGCAGCACAG CTGTCCGGGG ATCGCTGCAC GCTGAGCTCC CTCGGCAAGA 480

CCCAGCGGCG GCTCGGGATT TTTTTGGG 508

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 510 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..510
(D) OTHER INFORMATION: /note= "1 to 510 is -237 to 272 of Figure 5 Hsgr-21bf"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AATCTGGCCT CGGAACACGC CATTCTCCGC GCCGCTTCCA ATAACCACTA ACATCCCTAA 60

CGAGCATCCG AGCCGAGGGC TCTGCTCGGA AATCGTCCTG GCCCAACTCG GCCCTTCGAG 120

CTCTCGAAGA TTACCGCATC TATTTTTTTT TTCTTTTTTT TCTTTTCCTA GCGCAGATAA 180

AGTGAGCCCG GAAAGGGAAG GAGGGGGCGG GGACACCATT GCCCTGAAAG AATAAATAAG 240

TAAATAAACA AACTGGCTCC TCGCCGCAGC TGGACGCGGT CGGTTGAGTC CAGGTTGGGT 300

CGGACCTGAA CCCCTAAAAG CGGAACCGCC TCCCGCCCTC GCCATCCCGG AGCTGAGTCG 360

CCGGCGGCGG TGGCTGCTGC CAGACCCGGA GTTTCCTCTT TCACTGGATG GAGCTGAACT 420

TTGGGCGGCC AGAGCAGCAC AGCTGTCCGG GGATCGCTGC ACGCTGAGCT CCCTCGGCAA 480

GACCCAGCGG CGGCTCGGGA TTTTTTTGGG 510

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1927 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 538..1926

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..537
(D) OTHER INFORMATION: /note= "1 to 537 is -235 to 301 of Figure 5 21acon"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCTGGCCTCG GAACACGCCA TTCTCCGCGC CGCTTCCAAT AACCACTAAC ATCCCTAACG 60

AGCATCCGAG CCGAGGGCTC TGCTCGGAAA TCGTCCTGGC CCAACTCGGC CCTTCGAGCT 120

CTCGAAGATT ACCGCATCTA TTTTTTTTTT CTTTTTTTTC TTTTCCTAGC GCAGATAAAG 180

TGAGCCCGGA AAGGGAAGGA GGGGGCGGGG ACACCATTGC CCTGAAAGAA TAAATAAGTA 240

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AATAAACAAA CTGGCTCCTC GCCGCAGCTG GACGCGGTCG GTTGAGTCCA GGTTGGGTCG      300

GACCTGAACC CCTAAAAGCG GAACCGCCTC CCGCCCTCGC CATCCCGGAG CTGAGTCGCC      360

GGCGGCGGTG GCTGCTGCCA GACCCGGAGT TTCCTCTTTC ACTGGATGGA GCTGAACTTT      420

GGGCGGCCAG AGCAGCACAG CTGTCCGGGG ATCGCTGCAC GCTGAGCTCC CTCGGCAAGA      480

CCCAGCGGCG GCTCGGGATT TTTTTGGGGG GGCGGGGACC AGCCCCGCGC CGGCACC         537

ATG TTC CTG GCG NCC CTG TAC TTC GCG CTG CCG CTC TTG GAC TTG CTC         585
Met Phe Leu Ala Xaa Leu Tyr Phe Ala Leu Pro Leu Leu Asp Leu Leu
  1               5                  10                  15

CTG TCG GCC GAA GTG AGC GGC GGA GAC CGC CTG GAT TGC GTG AAA GCC         633
Leu Ser Ala Glu Val Ser Gly Gly Asp Arg Leu Asp Cys Val Lys Ala
            20                  25                  30

AGT GAT CAG TGC CTG AAG GAG CAG AGC TGC AGC ACC AAG TAC CGC ACG         681
Ser Asp Gln Cys Leu Lys Glu Gln Ser Cys Ser Thr Lys Tyr Arg Thr
        35                  40                  45

CTA AGG CAG TGC GTG GCG GGC AAG GAG ACC AAC TTC AGC CTG GCA TCC         729
Leu Arg Gln Cys Val Ala Gly Lys Glu Thr Asn Phe Ser Leu Ala Ser
    50                  55                  60

GGC CTG GAG GCC AAG GAT GAG TGC CGC AGC GCC ATG GAG GCC CTG AAG         777
Gly Leu Glu Ala Lys Asp Glu Cys Arg Ser Ala Met Glu Ala Leu Lys
65                  70                  75                  80

CAG AAG TCG CTC TAC AAC TGC CGC TGC AAG CGG GGT ATG AAG AAG GAG         825
Gln Lys Ser Leu Tyr Asn Cys Arg Cys Lys Arg Gly Met Lys Lys Glu
                85                  90                  95

AAG AAC TGC CTG CGC ATT TAC TGG AGC ATG TAC CAG AGC CTG CAG GGA         873
Lys Asn Cys Leu Arg Ile Tyr Trp Ser Met Tyr Gln Ser Leu Gln Gly
            100                 105                 110

AAT GAT CTG CTG GAG GAT TCC CCA TAT GAA CCA GTT AAC AGC AGA TTG         921
Asn Asp Leu Leu Glu Asp Ser Pro Tyr Glu Pro Val Asn Ser Arg Leu
        115                 120                 125

TCA GAT ATA TTC CGG GTG GTC CCA TTC ATA TCA GAT GTT TTT CAG CAA         969
Ser Asp Ile Phe Arg Val Val Pro Phe Ile Ser Asp Val Phe Gln Gln
    130                 135                 140

GTG GAG CAC ATT CCC AAA GGG AAC AAC TGC CTG GAT GCA GCG AAG GCC        1017
Val Glu His Ile Pro Lys Gly Asn Asn Cys Leu Asp Ala Ala Lys Ala
145                 150                 155                 160

TGC AAC CTC GAC GAC ATT TGC AAG AAG TAC AGG TCG GCG TAC ATC ACC        1065
Cys Asn Leu Asp Asp Ile Cys Lys Lys Tyr Arg Ser Ala Tyr Ile Thr
                165                 170                 175
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CCG TGC ACC ACC AGC GTG TCC AAC GAT GTC TGC AAC CGC CGC AAG TGC      1113
Pro Cys Thr Thr Ser Val Ser Asn Asp Val Cys Asn Arg Arg Lys Cys
            180                 185                 190

CAC AAG GCC CTC CGG CAG TTC TTT GAC AAG GTC CCG GCC AAG CAC AGC      1161
His Lys Ala Leu Arg Gln Phe Phe Asp Lys Val Pro Ala Lys His Ser
        195                 200                 205

TAC GGA ATG CTC TTC TGC TCC TGC CGG GAC ATC GCC TGC ACA GAG CGG      1209
Tyr Gly Met Leu Phe Cys Ser Cys Arg Asp Ile Ala Cys Thr Glu Arg
    210                 215                 220

AGG CGA CAG ACC ATC GTG CCT GTG TGC TCC TAT GAA GAG AGG GAG AAG      1257
Arg Arg Gln Thr Ile Val Pro Val Cys Ser Tyr Glu Glu Arg Glu Lys
225                 230                 235                 240

CCC AAC TGT TTG AAT TTG CAG GAC TCC TGC AAG ACG AAT TAC ATC TGC      1305
Pro Asn Cys Leu Asn Leu Gln Asp Ser Cys Lys Thr Asn Tyr Ile Cys
                245                 250                 255

AGA TCT CGC CTT GCG GAT TTT TTT ACC AAC TGC CAG CCA GAG TCA AGG      1353
Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys Gln Pro Glu Ser Arg
            260                 265                 270

TCT GTC AGC AGC TGT CTA AAG GAA AAC TAC GCT GAC TGC CTC CTC GCC      1401
Ser Val Ser Ser Cys Leu Lys Glu Asn Tyr Ala Asp Cys Leu Leu Ala
        275                 280                 285

TAC TCG GGG CTT ATT GGC ACA GTC ATG ACC CCC AAC TAC ATA GAC TCC      1449
Tyr Ser Gly Leu Ile Gly Thr Val Met Thr Pro Asn Tyr Ile Asp Ser
    290                 295                 300

AGT AGC CTC AGT GTG GCC CCA TGG TGT GAC TGC AGC AAC AGT GGG AAC      1497
Ser Ser Leu Ser Val Ala Pro Trp Cys Asp Cys Ser Asn Ser Gly Asn
305                 310                 315                 320

GAC CTA GAA GAG TGC TTG AAA TTT TTG AAT TTC TTC AAG GAC AAT ACA      1545
Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn Thr
                325                 330                 335

TGT CTT AAA AAT GCA ATT CAA GCC TTT GGC AAT GGC TCC GAT GTG ACC      1593
Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp Val Thr
            340                 345                 350

GTG TGG CAG CCA GCC TTC CCA GTA CAG ACC ACC ACT GCC ACT ACC ACC      1641
Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr Thr Ala Thr Thr Thr
        355                 360                 365

ACT GCC CTC CGG GTT AAG AAC AAG CCC CTG GGG CCA GCA GGG TCT GAG      1689
Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser Glu
    370                 375                 380

AAT GAA ATT CCC ACT CAT GTT TTG CCA CCG TGT GCA AAT TTA CAG GCA      1737
Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln Ala
385                 390                 395                 400
```

```
CAG AAG CTG AAA TCC AAT GTG TCG GGC AAT ACA CAC CTC TGT ATT TCC      1785
Gln Lys Leu Lys Ser Asn Val Ser Gly Asn Thr His Leu Cys Ile Ser
                405                 410                 415

AAT GGT AAT TAT GAA AAA GAA GGT CTC GGT GCT TCC AGC CAC ATA ACC      1833
Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser His Ile Thr
            420                 425                 430

ACA AAA TCA ATG GCT GCT CCT CCA AGC TGT GGT CTG AGC CCA CTG CTG      1881
Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro Leu Leu
        435                 440                 445

GTC CTG GTG GTA ACC GCT CTG TCC ACC CTA TTA TCT TTA ACA GAA          1926
Val Leu Val Val Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr Glu
    450                 455                 460

A                                                                    1927
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 463 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Phe Leu Ala Xaa Leu Tyr Phe Ala Leu Pro Leu Leu Asp Leu Leu
  1               5                  10                  15

Leu Ser Ala Glu Val Ser Gly Gly Asp Arg Leu Asp Cys Val Lys Ala
            20                  25                  30

Ser Asp Gln Cys Leu Lys Glu Gln Ser Cys Ser Thr Lys Tyr Arg Thr
        35                  40                  45

Leu Arg Gln Cys Val Ala Gly Lys Glu Thr Asn Phe Ser Leu Ala Ser
    50                  55                  60

Gly Leu Glu Ala Lys Asp Glu Cys Arg Ser Ala Met Glu Ala Leu Lys
65                  70                  75                  80

Gln Lys Ser Leu Tyr Asn Cys Arg Cys Lys Arg Gly Met Lys Lys Glu
                85                  90                  95

Lys Asn Cys Leu Arg Ile Tyr Trp Ser Met Tyr Gln Ser Leu Gln Gly
            100                 105                 110

Asn Asp Leu Leu Glu Asp Ser Pro Tyr Glu Pro Val Asn Ser Arg Leu
        115                 120                 125

Ser Asp Ile Phe Arg Val Val Pro Phe Ile Ser Asp Val Phe Gln Gln
    130                 135                 140
```

```
Val Glu His Ile Pro Lys Gly Asn Asn Cys Leu Asp Ala Ala Lys Ala
145                 150                 155                 160

Cys Asn Leu Asp Asp Ile Cys Lys Lys Tyr Arg Ser Ala Tyr Ile Thr
                165                 170                 175

Pro Cys Thr Thr Ser Val Ser Asn Asp Val Cys Asn Arg Arg Lys Cys
            180                 185                 190

His Lys Ala Leu Arg Gln Phe Phe Asp Lys Val Pro Ala Lys His Ser
        195                 200                 205

Tyr Gly Met Leu Phe Cys Ser Cys Arg Asp Ile Ala Cys Thr Glu Arg
    210                 215                 220

Arg Arg Gln Thr Ile Val Pro Val Cys Ser Tyr Glu Glu Arg Glu Lys
225                 230                 235                 240

Pro Asn Cys Leu Asn Leu Gln Asp Ser Cys Lys Thr Asn Tyr Ile Cys
                245                 250                 255

Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys Gln Pro Glu Ser Arg
            260                 265                 270

Ser Val Ser Ser Cys Leu Lys Glu Asn Tyr Ala Asp Cys Leu Leu Ala
        275                 280                 285

Tyr Ser Gly Leu Ile Gly Thr Val Met Thr Pro Asn Tyr Ile Asp Ser
    290                 295                 300

Ser Ser Leu Ser Val Ala Pro Trp Cys Asp Cys Ser Asn Ser Gly Asn
305                 310                 315                 320

Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn Thr
                325                 330                 335

Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp Val Thr
            340                 345                 350

Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr Thr Ala Thr Thr Thr
        355                 360                 365

Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser Glu
    370                 375                 380

Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln Ala
385                 390                 395                 400

Gln Lys Leu Lys Ser Asn Val Ser Gly Asn Thr His Leu Cys Ile Ser
                405                 410                 415

Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser His Ile Thr
            420                 425                 430
```

```
Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro Leu Leu
        435                 440                 445

Val Leu Val Val Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr Glu
    450                 455                 460
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1929 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 540..1928

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..539
(D) OTHER INFORMATION: /note= "1 to 539 is -237 to 301 of Figure 5 21bcon"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
AATCTGGCCT CGGAACACGC CATTCTCCGC GCCGCTTCCA ATAACCACTA ACATCCCTAA      60

CGAGCATCCG AGCCGAGGGC TCTGCTCGGA AATCGTCCTG GCCCAACTCG GCCCTTCGAG     120

CTCTCGAAGA TTACCGCATC TATTTTTTTT TTCTTTTTTT TCTTTTCCTA GCGCAGATAA     180

AGTGAGCCCG GAAAGGGAAG GAGGGGGCGG GGACACCATT GCCCTGAAAG AATAAATAAG     240

TAAATAAACA AACTGGCTCC TCGCCGCAGC TGGACGCGGT CGGTTGAGTC CAGGTTGGGT     300

CGGACCTGAA CCCCTAAAAG CGGAACCGCC TCCCGCCCTC GCCATCCCGG AGCTGAGTCG     360

CCGGCGGCGG TGGCTGCTGC CAGACCCGGA GTTTCCTCTT TCACTGGATG GAGCTGAACT     420

TTGGGCGGCC AGAGCAGCAC AGCTGTCCGG GGATCGCTGC ACGCTGAGCT CCCTCGGCAA     480

GACCCAGCGG CGGCTCGGGA TTTTTTTGGG GGGGCGGGGA CCAGCCCCGC GCCGGCACC      539

ATG TTC CTG GCG ACC CTG TAC TTC GCG CTG CCG CTC TTG GAC TTG CTC        587
Met Phe Leu Ala Thr Leu Tyr Phe Ala Leu Pro Leu Leu Asp Leu Leu
  1               5                  10                  15

CTG TCG GCC GAA GTG AGC GGC GGA GAC CGC CTG GAT TGC GTG AAA GCC       635
Leu Ser Ala Glu Val Ser Gly Gly Asp Arg Leu Asp Cys Val Lys Ala
            20                  25                  30
```

```
AGT GAT CAG TGC CTG AAG GAG CAG AGC TGC AGC ACC AAG TAC CGC ACG        683
Ser Asp Gln Cys Leu Lys Glu Gln Ser Cys Ser Thr Lys Tyr Arg Thr
          35                  40                  45

CTA AGG CAG TGC GTG GCG GGC AAG GAG ACC AAC TTC AGC CTG GCA TCC        731
Leu Arg Gln Cys Val Ala Gly Lys Glu Thr Asn Phe Ser Leu Ala Ser
     50                  55                  60

GGC CTG GAG GCC AAG GAT GAG TGC CGC AGC GCC ATG GAG GCC CTG AAG        779
Gly Leu Glu Ala Lys Asp Glu Cys Arg Ser Ala Met Glu Ala Leu Lys
 65                  70                  75                  80

CAG AAG TCG CTC TAC AAC TGC CGC TGC AAG CGG GGT ATG AAG AAG GAG        827
Gln Lys Ser Leu Tyr Asn Cys Arg Cys Lys Arg Gly Met Lys Lys Glu
                 85                  90                  95

AAG AAC TGC CTG CGC ATT TAC TGG AGC ATG TAC CAG AGC CTG CAG GGA        875
Lys Asn Cys Leu Arg Ile Tyr Trp Ser Met Tyr Gln Ser Leu Gln Gly
            100                 105                 110

AAT GAT CTG CTG GAG GAT TCC CCA TAT GAA CCA GTT AAC AGC AGA TTG        923
Asn Asp Leu Leu Glu Asp Ser Pro Tyr Glu Pro Val Asn Ser Arg Leu
        115                 120                 125

TCA GAT ATA TTC CGG GTG GTC CCA TTC ATA TCA GAT GTT TTT CAG CAA        971
Ser Asp Ile Phe Arg Val Val Pro Phe Ile Ser Asp Val Phe Gln Gln
    130                 135                 140

GTG GAG CAC ATT CCC AAA GGG AAC AAC TGC CTG GAT GCA GCG AAG GCC       1019
Val Glu His Ile Pro Lys Gly Asn Asn Cys Leu Asp Ala Ala Lys Ala
145                 150                 155                 160

TGC AAC CTC GAC GAC ATT TGC AAG AAG TAC AGG TCG GCG TAC ATC ACC       1067
Cys Asn Leu Asp Asp Ile Cys Lys Lys Tyr Arg Ser Ala Tyr Ile Thr
                165                 170                 175

CCG TGC ACC ACC AGC GTG TCC AAC GAT GTC TGC AAC CGC CGC AAG TGC       1115
Pro Cys Thr Thr Ser Val Ser Asn Asp Val Cys Asn Arg Arg Lys Cys
            180                 185                 190

CAC AAG GCC CTC CGG CAG TTC TTT GAC AAG GTC CCG GCC AAG CAC AGC       1163
His Lys Ala Leu Arg Gln Phe Phe Asp Lys Val Pro Ala Lys His Ser
        195                 200                 205

TAC GGA ATG CTC TTC TGC TCC TGC CGG GAC ATC GCC TGC ACA GAG CGG       1211
Tyr Gly Met Leu Phe Cys Ser Cys Arg Asp Ile Ala Cys Thr Glu Arg
    210                 215                 220

AGG CGA CAG ACC ATC GTG CCT GTG TGC TCC TAT GAA GAG AGG GAG AAG       1259
Arg Arg Gln Thr Ile Val Pro Val Cys Ser Tyr Glu Glu Arg Glu Lys
225                 230                 235                 240

CCC AAC TGT TTG AAT TTG CAG GAC TCC TGC AAG ACG AAT TAC ATC TGC       1307
Pro Asn Cys Leu Asn Leu Gln Asp Ser Cys Lys Thr Asn Tyr Ile Cys
                245                 250                 255
```

AGA TCT CGC CTT GCG GAT TTT TTT ACC AAC TGC CAG CCA GAG TCA AGG 1355
Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys Gln Pro Glu Ser Arg
260 265 270

TCT GTC AGC AGC TGT CTA AAG GAA AAC TAC GCT GAC TGC CTC CTC GCC 1403
Ser Val Ser Ser Cys Leu Lys Glu Asn Tyr Ala Asp Cys Leu Leu Ala
275 280 285

TAC TCG GGG CTT ATT GGC ACA GTC ATG ACC CCC AAC TAC ATA GAC TCC 1451
Tyr Ser Gly Leu Ile Gly Thr Val Met Thr Pro Asn Tyr Ile Asp Ser
290 295 300

AGT AGC CTC AGT GTG GCC CCA TGG TGT GAC TGC AGC AAC AGT GGG AAC 1499
Ser Ser Leu Ser Val Ala Pro Trp Cys Asp Cys Ser Asn Ser Gly Asn
305 310 315 320

GAC CTA GAA GAG TGC TTG AAA TTT TTG AAT TTC TTC AAG GAC AAT ACA 1547
Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn Thr
325 330 335

TGT CTT AAA AAT GCA ATT CAA GCC TTT GGC AAT GGC TCC GAT GTG ACC 1595
Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp Val Thr
340 345 350

GTG TGG CAG CCA GCC TTC CCA GTA CAG ACC ACC ACT GCC ACT ACC ACC 1643
Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr Thr Ala Thr Thr Thr
355 360 365

ACT GCC CTC CGG GTT AAG AAC AAG CCC CTG GGG CCA GCA GGG TCT GAG 1691
Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser Glu
370 375 380

AAT GAA ATT CCC ACT CAT GTT TTG CCA CCG TGT GCA AAT TTA CAG GCA 1739
Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln Ala
385 390 395 400

CAG AAG CTG AAA TCC AAT GTG TCG GGC AAT ACA CAC CTC TGT ATT TCC 1787
Gln Lys Leu Lys Ser Asn Val Ser Gly Asn Thr His Leu Cys Ile Ser
405 410 415

AAT GGT AAT TAT GAA AAA GAA GGT CTC GGT GCT TCC AGC CAC ATA ACC 1835
Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser His Ile Thr
420 425 430

ACA AAA TCA ATG GCT GCT CCT CCA AGC TGT GGT CTG AGC CCA CTG CTG 1883
Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro Leu Leu
435 440 445

GTC CTG GTG GTA ACC GCT CTG TCC ACC CTA TTA TCT TTA ACA GAA 1928
Val Leu Val Val Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr Glu
450 455 460

A 1929

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 463 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Met Phe Leu Ala Thr Leu Tyr Phe Ala Leu Pro Leu Leu Asp Leu Leu
 1               5                  10                  15

Leu Ser Ala Glu Val Ser Gly Gly Asp Arg Leu Asp Cys Val Lys Ala
            20                  25                  30

Ser Asp Gln Cys Leu Lys Glu Gln Ser Cys Ser Thr Lys Tyr Arg Thr
        35                  40                  45

Leu Arg Gln Cys Val Ala Gly Lys Glu Thr Asn Phe Ser Leu Ala Ser
    50                  55                  60

Gly Leu Glu Ala Lys Asp Glu Cys Arg Ser Ala Met Glu Ala Leu Lys
65                  70                  75                  80

Gln Lys Ser Leu Tyr Asn Cys Arg Cys Lys Arg Gly Met Lys Lys Glu
                85                  90                  95

Lys Asn Cys Leu Arg Ile Tyr Trp Ser Met Tyr Gln Ser Leu Gln Gly
            100                 105                 110

Asn Asp Leu Leu Glu Asp Ser Pro Tyr Glu Pro Val Asn Ser Arg Leu
        115                 120                 125

Ser Asp Ile Phe Arg Val Val Pro Phe Ile Ser Asp Val Phe Gln Gln
    130                 135                 140

Val Glu His Ile Pro Lys Gly Asn Asn Cys Leu Asp Ala Ala Lys Ala
145                 150                 155                 160

Cys Asn Leu Asp Asp Ile Cys Lys Lys Tyr Arg Ser Ala Tyr Ile Thr
                165                 170                 175

Pro Cys Thr Thr Ser Val Ser Asn Asp Val Cys Asn Arg Arg Lys Cys
            180                 185                 190

His Lys Ala Leu Arg Gln Phe Phe Asp Lys Val Pro Ala Lys His Ser
        195                 200                 205

Tyr Gly Met Leu Phe Cys Ser Cys Arg Asp Ile Ala Cys Thr Glu Arg
    210                 215                 220

Arg Arg Gln Thr Ile Val Pro Val Cys Ser Tyr Glu Glu Arg Glu Lys
225                 230                 235                 240
```

```
Pro Asn Cys Leu Asn Leu Gln Asp Ser Cys Lys Thr Asn Tyr Ile Cys
                245                 250                 255

Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys Gln Pro Glu Ser Arg
            260                 265                 270

Ser Val Ser Ser Cys Leu Lys Glu Asn Tyr Ala Asp Cys Leu Leu Ala
        275                 280                 285

Tyr Ser Gly Leu Ile Gly Thr Val Met Thr Pro Asn Tyr Ile Asp Ser
    290                 295                 300

Ser Ser Leu Ser Val Ala Pro Trp Cys Asp Cys Ser Asn Ser Gly Asn
305                 310                 315                 320

Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn Thr
                325                 330                 335

Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp Val Thr
            340                 345                 350

Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr Thr Ala Thr Thr Thr
        355                 360                 365

Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser Glu
    370                 375                 380

Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln Ala
385                 390                 395                 400

Gln Lys Leu Lys Ser Asn Val Ser Gly Asn Thr His Leu Cys Ile Ser
                405                 410                 415

Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser His Ile Thr
            420                 425                 430

Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro Leu Leu
        435                 440                 445

Val Leu Val Val Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr Glu
    450                 455                 460
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 699 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..699

(D) OTHER INFORMATION: /note= "1 to 699 is 814 to 1512 of Figure 5 Hsgr-29a"

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..697

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
G TCG GCG TAC ATC ACC CCG TGC ACC ACC AGC GTG TCC AAT GAT GTC          46
  Ser Ala Tyr Ile Thr Pro Cys Thr Thr Ser Val Ser Asn Asp Val
    1               5                  10                  15

TGC AAC CGC CGC AAG TGC CAC AAG GCC CTC CGG CAG TTC TTT GAC AAG        94
Cys Asn Arg Arg Lys Cys His Lys Ala Leu Arg Gln Phe Phe Asp Lys
                20                  25                  30

GTC CCG GCC AAG CAC AGC TAC GGA ATG CTC TTC TGC TCC TGC CGG GAC       142
Val Pro Ala Lys His Ser Tyr Gly Met Leu Phe Cys Ser Cys Arg Asp
            35                  40                  45

ATC GCC TGC ACA GAG CGG AGG CGA CAG ACC ATC GTG CCT GTG TGC TCC       190
Ile Ala Cys Thr Glu Arg Arg Arg Gln Thr Ile Val Pro Val Cys Ser
        50                  55                  60

TAT GAA GAG AGG GAG AAG CCC AAC TGT TTG AAT TTG CAG GAC TCC TGC       238
Tyr Glu Glu Arg Glu Lys Pro Asn Cys Leu Asn Leu Gln Asp Ser Cys
    65                  70                  75

AAG ACG AAT TAC ATC TGC AGA TCT CGC CTT GCG GAT TTT TTT ACC AAC       286
Lys Thr Asn Tyr Ile Cys Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn
80                  85                  90                  95

TGC CAG CCA GAG TCA AGG TCT GTC AGC AGC TGT CTA AAG GAA AAC TAC       334
Cys Gln Pro Glu Ser Arg Ser Val Ser Ser Cys Leu Lys Glu Asn Tyr
                100                 105                 110

GCT GAC TGC CTC CTC GCC TAC TCG GGG CTT ATT GGC ACA GTC ATG ACC       382
Ala Asp Cys Leu Leu Ala Tyr Ser Gly Leu Ile Gly Thr Val Met Thr
            115                 120                 125

CCC AAC TAC ATA GAC TCC AGT AGC CTC AGT GTG GCC CCA TGG TGT GAC       430
Pro Asn Tyr Ile Asp Ser Ser Ser Leu Ser Val Ala Pro Trp Cys Asp
        130                 135                 140

TGC AGC AAC AGT GGG AAC GAC CTA GAA GAG TGC TTG AAA TTT TTG AAT       478
Cys Ser Asn Ser Gly Asn Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn
    145                 150                 155

TTC TTC AAG GAC AAT ACA TGT CTT AAA AAT GCA ATT CAA GCC TTT GGC       526
Phe Phe Lys Asp Asn Thr Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly
160                 165                 170                 175

AAT GGC TCC GAT GTG ACC GTG TGG CAG CCA GCC TTC CCA GTA CAG ACC       574
Asn Gly Ser Asp Val Thr Val Trp Gln Pro Ala Phe Pro Val Gln Thr
            180                 185                 190
```

```
ACC ACT GCC GCT ACC ACC ACT GCC CTC CGG GTT AAG AAC AAG CCC CTG          622
Thr Thr Ala Ala Thr Thr Thr Ala Leu Arg Val Lys Asn Lys Pro Leu
            195                 200                     205

GGG CCA GCA GGG TCT GAG AAT GAA ATT CCC ACT CAT GTT TTG CCA CCG          670
Gly Pro Ala Gly Ser Glu Asn Glu Ile Pro Thr His Val Leu Pro Pro
        210                 215                     220

TGT GCA AAT TTA CAG GCA CAG AAG CTG AA                                   699
Cys Ala Asn Leu Gln Ala Gln Lys Leu
    225                 230
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 232 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Ser Ala Tyr Ile Thr Pro Cys Thr Thr Ser Val Ser Asn Asp Val Cys
 1               5                  10                  15

Asn Arg Arg Lys Cys His Lys Ala Leu Arg Gln Phe Phe Asp Lys Val
            20                  25                  30

Pro Ala Lys His Ser Tyr Gly Met Leu Phe Cys Ser Cys Arg Asp Ile
        35                  40                  45

Ala Cys Thr Glu Arg Arg Arg Gln Thr Ile Val Pro Val Cys Ser Tyr
    50                  55                  60

Glu Glu Arg Glu Lys Pro Asn Cys Leu Asn Leu Gln Asp Ser Cys Lys
65                  70                  75                  80

Thr Asn Tyr Ile Cys Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys
                85                  90                  95

Gln Pro Glu Ser Arg Ser Val Ser Ser Cys Leu Lys Glu Asn Tyr Ala
            100                 105                 110

Asp Cys Leu Leu Ala Tyr Ser Gly Leu Ile Gly Thr Val Met Thr Pro
        115                 120                 125

Asn Tyr Ile Asp Ser Ser Ser Leu Ser Val Ala Pro Trp Cys Asp Cys
    130                 135                 140

Ser Asn Ser Gly Asn Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe
145                 150                 155                 160

Phe Lys Asp Asn Thr Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn
                165                 170                 175
```

```
Gly Ser Asp Val Thr Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr
            180                 185                 190

Thr Ala Ala Thr Thr Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly
        195                 200                 205

Pro Ala Gly Ser Glu Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys
    210                 215                 220

Ala Asn Leu Gln Ala Gln Lys Leu
225                 230
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2157 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..886

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..2157
(D) OTHER INFORMATION: /note= "1 to 2157 is 814 to 2971 of Figure 5 29brc"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
G TCG GCG TAC ATC ACC CCG TGC ACC ACC AGC GTG TCC AAT GAT GTC          46
  Ser Ala Tyr Ile Thr Pro Cys Thr Thr Ser Val Ser Asn Asp Val
    1               5                  10                  15

TGC AAC CGC CGC AAG TGC CAC AAG GCC CTC CGG CAG TTC TTT GAC AAG        94
Cys Asn Arg Arg Lys Cys His Lys Ala Leu Arg Gln Phe Phe Asp Lys
                20                  25                  30

GTC CCG GCC AAG CAC AGC TAC GGA ATG CTC TTC TGC TCC TGC CGG GAC       142
Val Pro Ala Lys His Ser Tyr Gly Met Leu Phe Cys Ser Cys Arg Asp
            35                  40                  45

ATC GCC TGC ACA GAG CGG AGG CGA CAG ACC ATC GTG CCT GTG TGC TCC       190
Ile Ala Cys Thr Glu Arg Arg Arg Gln Thr Ile Val Pro Val Cys Ser
        50                  55                  60

TAT GAA GAG AGG GAG AAG CCC AAC TGT TTG AAT TTG CAG GAC TCC TGC       238
Tyr Glu Glu Arg Glu Lys Pro Asn Cys Leu Asn Leu Gln Asp Ser Cys
    65                  70                  75
```

```
AAG ACG AAT TAC ATC TGC AGA TCT CGC CTT GCG GAT TTT TTT ACC AAC        286
Lys Thr Asn Tyr Ile Cys Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn
 80                 85                  90                  95

TGC CAG CCA GAG TCA AGG TCT GTC AGC AGC TGT CTA AAG GAA AAC TAC        334
Cys Gln Pro Glu Ser Arg Ser Val Ser Ser Cys Leu Lys Glu Asn Tyr
            100                 105                 110

GCT GAC TGC CTC CTC GCC TAC TCG GGG CTT ATT GGC ACA GTC ATG ACC        382
Ala Asp Cys Leu Leu Ala Tyr Ser Gly Leu Ile Gly Thr Val Met Thr
        115                 120                 125

CCC AAC TAC ATA GAC TCC AGT AGC CTC AGT GTG GCC CCA TGG TGT GAC        430
Pro Asn Tyr Ile Asp Ser Ser Ser Leu Ser Val Ala Pro Trp Cys Asp
    130                 135                 140

TGC AGC AAC AGT GGG AAC GAC CTA GAA GAG TGC TTG AAA TTT TTG AAT        478
Cys Ser Asn Ser Gly Asn Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn
    145                 150                 155

TTC TTC AAG GAC AAT ACA TGT CTT AAA AAT GCA ATT CAA GCC TTT GGC        526
Phe Phe Lys Asp Asn Thr Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly
160                 165                 170                 175

AAT GGC TCC GAT GTG ACC GTG TGG CAG CCA GCC TTC CCA GTA CAG ACC        574
Asn Gly Ser Asp Val Thr Val Trp Gln Pro Ala Phe Pro Val Gln Thr
            180                 185                 190

ACC ACT GCC GCT ACC ACC ACT GCC CTC CGG GTT AAG AAC AAG CCC CTG        622
Thr Thr Ala Ala Thr Thr Thr Ala Leu Arg Val Lys Asn Lys Pro Leu
        195                 200                 205

GGG CCA GCA GGG TCT GAG AAT GAA ATT CCC ACT CAT GTT TTG CCA CCG        670
Gly Pro Ala Gly Ser Glu Asn Glu Ile Pro Thr His Val Leu Pro Pro
    210                 215                 220

TGT GCA AAT TTA CAG GCA CAG AAG CTG AAA TCC AAT GTG TCG GGC AAT        718
Cys Ala Asn Leu Gln Ala Gln Lys Leu Lys Ser Asn Val Ser Gly Asn
    225                 230                 235

ACA CAC CTC TGT ATT TCC AAT GGT AAT TAT GAA AAA GAA GGT CTC GGT        766
Thr His Leu Cys Ile Ser Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly
240                 245                 250                 255

GCT TCC AGC CAC ATA ACC ACA AAA TCA ATG GCT GCT CCT CCA AGC TGT        814
Ala Ser Ser His Ile Thr Thr Lys Ser Met Ala Ala Pro Pro Ser Cys
            260                 265                 270

GGT CTG AGC CCA CTG CTG GTC CTG GTG GTA ACC GCT CTG TCC ACC CTA        862
Gly Leu Ser Pro Leu Leu Val Leu Val Val Thr Ala Leu Ser Thr Leu
        275                 280                 285

TTA TCT TTA ACA GAA ACA TCA TAG CTGCATTAAA AAAATACAAT ATGGACATGT       916
Leu Ser Leu Thr Glu Thr Ser  *
    290                 295
```

AAAAAGACAA AAACCAAGTT ATCTGTTTCC TGTTCTCTTG TATAGCTGAA ATTCCAGTTT 976

AGGAGCTCAG TTGAGAAACA GTTCCATTCA ACTGGAACAT TTTTTTTTTT CCTTTTAAGA 1036

AAGCTTCTTG TGATCCTTCG GGGCTTCTGT GAAAAACCTG ATGCAGTGCT CCATCCAAAC 1096

TCAGAAGGCT TTGGGATATG CTGTATTTTA AAGGGACAGT TTGTAACTTG GGCTGTAAAG 1156

CAAACTGGGG CTGTGTTTTC GATGATGATG ATCATCATGA TCATGATNNN NNNNNNNNNN 1216

NNNNNNNNNN NNNNNNNNNN NNNNNNGATT TTAACAGTTT TACTTCTGGC CTTTCCTAGC 1276

TAGAGAAGGA GTTAATATTT CTAAGGTAAC TCCCATATCT CCTTTAATGA CATTGATTTC 1336

TAATGATATA AATTTCAGCC TACATTGATG CCAAGCTTTT TTGCCACAAA GAAGATTCTT 1396

ACCAAGAGTG GGCTTTGTGG AAACAGCTGG TACTGATGTT CACCTTTATA TATGTACTAG 1456

CATTTTCCAC GCTGATGTTT ATGTACTGTA AACAGTTCTG CACTCTTGTA CAAAAGAAAA 1516

AACACCTGTC ACATCCAAAT ATAGTATCTG TCTTTTCGTC AAAATAGAGA GTGGGGAATG 1576

AGTGTGCCGA TTCAATACCT CAATCCCTGA ACGACACTCT CCTAATCCTA AGCCTTACCT 1636

GAGTGAGAAG CCCTTTACCT AACAAAAGTC CAATATAGCT GAAATGTCGC TCTAATACTC 1696

TTTACACATA TGAGGTTATA TGTAGAAAAA AATTTTACTA CTAAATGATT TCAACTATTG 1756

GCTTTCTATA TTTTGAAAGT AATGATATTG TCTCATTTTT TTACTGATGG TTTAATACAA 1816

AATACACAGA GCTTGTTTCC CCTCATAAGT AGTGTTCGCT CTGATATGAA CTTCACAAAT 1876

ACAGCTCATC AAAAGCAGAC TCTGAGAAGC CTCGTGCTGT AGCAGAAAGT TCTGCATCAT 1936

GTGACTGTGG ACAGGCAGGA GGAAACAGAA CAGACAAGCA TTGTCTTTTG TCATTGCTCG 1996

AAGTGCAAGC GTGCATACCT GTGGAGGGAA CTGGTGGCTG CTTGTAAATG TTCTGCAGCA 2056

TCTCTTGACA CACTTGTCAT GACACAATCC AGTACCTTGG TTTTCAGGTT ATCTGACAAA 2116

GGCAGCTTTG ATTGGGACAT GGAGGCATGG GCAGGCCGGA A 2157

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 295 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ser Ala Tyr Ile Thr Pro Cys Thr Thr Ser Val Ser Asn Asp Val Cys
1 5 10 15

Asn Arg Arg Lys Cys His Lys Ala Leu Arg Gln Phe Phe Asp Lys Val
20 25 30

Pro Ala Lys His Ser Tyr Gly Met Leu Phe Cys Ser Cys Arg Asp Ile
35 40 45

Ala Cys Thr Glu Arg Arg Arg Gln Thr Ile Val Pro Val Cys Ser Tyr
50 55 60

Glu Glu Arg Glu Lys Pro Asn Cys Leu Asn Leu Gln Asp Ser Cys Lys
65 70 75 80

Thr Asn Tyr Ile Cys Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys
85 90 95

Gln Pro Glu Ser Arg Ser Val Ser Ser Cys Leu Lys Glu Asn Tyr Ala
100 105 110

Asp Cys Leu Leu Ala Tyr Ser Gly Leu Ile Gly Thr Val Met Thr Pro
115 120 125

Asn Tyr Ile Asp Ser Ser Ser Leu Ser Val Ala Pro Trp Cys Asp Cys
130 135 140

Ser Asn Ser Gly Asn Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe
145 150 155 160

Phe Lys Asp Asn Thr Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn
165 170 175

Gly Ser Asp Val Thr Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr
180 185 190

Thr Ala Ala Thr Thr Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly
195 200 205

Pro Ala Gly Ser Glu Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys
210 215 220

Ala Asn Leu Gln Ala Gln Lys Leu Lys Ser Asn Val Ser Gly Asn Thr
225 230 235 240

His Leu Cys Ile Ser Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly Ala
245 250 255

Ser Ser His Ile Thr Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Gly
260 265 270

Leu Ser Pro Leu Leu Val Leu Val Val Thr Ala Leu Ser Thr Leu Leu
275 280 285

Ser Leu Thr Glu Thr Ser *
290 295

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 659 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..658

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..659
(D) OTHER INFORMATION: /note= "1 to 659 is 1033 to 1691 of Figure 5 Hsgr-21ar"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
G AAT TTG CAG GAC TCC TGC AAG ACG AAT TAC ATC TGC AGA TCT CGC          46
  Asn Leu Gln Asp Ser Cys Lys Thr Asn Tyr Ile Cys Arg Ser Arg
    1               5                  10                  15

CTT GCG GAT TTT TTT ACC AAC TGC CAG CCA GAG TCA AGG TCT GTC AGC        94
Leu Ala Asp Phe Phe Thr Asn Cys Gln Pro Glu Ser Arg Ser Val Ser
                20                  25                  30

AGC TGT CTA AAG GAA AAC TAC GCT GAC TGC CTC CTC GCC TAC TCG GGG       142
Ser Cys Leu Lys Glu Asn Tyr Ala Asp Cys Leu Leu Ala Tyr Ser Gly
            35                  40                  45

CTT ATT GGC ACA GTC ATG ACC CCC AAC TAC ATA GAC TCC AGT AGC CTC       190
Leu Ile Gly Thr Val Met Thr Pro Asn Tyr Ile Asp Ser Ser Ser Leu
        50                  55                  60

AGT GTG GCC CCA TGG TGT GAC TGC AGC AAC AGT GGG AAC GAC CTA GAA       238
Ser Val Ala Pro Trp Cys Asp Cys Ser Asn Ser Gly Asn Asp Leu Glu
    65                  70                  75

GAG TGC TTG AAA TTT TTG AAT TTC TTC AAG GAC AAT ACA TGT CTT AAA       286
Glu Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn Thr Cys Leu Lys
80                  85                  90                  95

AAT GCA ATT CAA GCC TTT GGC AAT GGC TCC GAT GTG ACC GTG TGG CAG       334
Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp Val Thr Val Trp Gln
                100                 105                 110

CCA GCC TTC CCA GTA CAG ACC ACC ACT GCC ACT ACC ACC ACT GCC CTC       382
Pro Ala Phe Pro Val Gln Thr Thr Thr Ala Thr Thr Thr Thr Ala Leu
            115                 120                 125
```

```
CGG GTT AAG AAC AAG CCC CTG GGG CCA GCA GGG TCT GAG AAT GAA ATT        430
Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser Glu Asn Glu Ile
        130                 135                 140

CCC ACT CAT GTT TTG CCA CCG TGT GCA AAT TTA CAG GCA CAG AAG CTG        478
Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln Ala Gln Lys Leu
    145                 150                 155

AAA TCC AAT GTG TCG GGC AAT ACA CAC CTC TGT ATT TCC AAT GGT AAT        526
Lys Ser Asn Val Ser Gly Asn Thr His Leu Cys Ile Ser Asn Gly Asn
160                 165                 170                 175

TAT GAA AAA GAA GGT CTC GGT GCT TCC AGC CAC ATA ACC ACA AAA TCA        574
Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser His Ile Thr Thr Lys Ser
                180                 185                 190

ATG GCT GCT CCT CCA AGC TGT GGT CTG AGC CCA CTG CTG GTC CTG GTG        622
Met Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro Leu Leu Val Leu Val
            195                 200                 205

GTA ACC GCT CTG TCC ACC CTA TTA TCT TTA ACA GAA A                      659
Val Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr Glu
        210                 215
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 219 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Asn Leu Gln Asp Ser Cys Lys Thr Asn Tyr Ile Cys Arg Ser Arg Leu
 1               5                  10                  15

Ala Asp Phe Phe Thr Asn Cys Gln Pro Glu Ser Arg Ser Val Ser Ser
            20                  25                  30

Cys Leu Lys Glu Asn Tyr Ala Asp Cys Leu Leu Ala Tyr Ser Gly Leu
        35                  40                  45

Ile Gly Thr Val Met Thr Pro Asn Tyr Ile Asp Ser Ser Ser Leu Ser
    50                  55                  60

Val Ala Pro Trp Cys Asp Cys Ser Asn Ser Gly Asn Asp Leu Glu Glu
65                  70                  75                  80

Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn Thr Cys Leu Lys Asn
                85                  90                  95

Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp Val Thr Val Trp Gln Pro
            100                 105                 110
```

```
Ala Phe Pro Val Gln Thr Thr Thr Ala Thr Thr Thr Thr Ala Leu Arg
        115                 120                 125

Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser Glu Asn Glu Ile Pro
    130                 135                 140

Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln Ala Gln Lys Leu Lys
145                 150                 155                 160

Ser Asn Val Ser Gly Asn Thr His Leu Cys Ile Ser Asn Gly Asn Tyr
                165                 170                 175

Glu Lys Glu Gly Leu Gly Ala Ser Ser His Ile Thr Thr Lys Ser Met
            180                 185                 190

Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro Leu Leu Val Leu Val Val
        195                 200                 205

Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr Glu
    210                 215
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 630 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..629

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..630
(D) OTHER INFORMATION: /note= "1 to 630 is 1062 to 1691 of Figure 5 Hsgr-21br"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
AC ATC TGC AGA TCT CGC CTT GCG GAT TTT TTT ACC AAC TGC CAG CCA          47
   Ile Cys Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys Gln Pro
    1               5                   10                  15

GAG TCA AGG TCT GTC AGC AGC TGT CTA AAG GAA AAC TAC GCT GAC TGC          95
Glu Ser Arg Ser Val Ser Ser Cys Leu Lys Glu Asn Tyr Ala Asp Cys
                20                  25                  30

CTC CTC GCC TAC TCG GGG CTT ATT GGC ACA GTC ATG ACC CCC AAC TAC         143
Leu Leu Ala Tyr Ser Gly Leu Ile Gly Thr Val Met Thr Pro Asn Tyr
            35                  40                  45
```

```
ATA GAC TCC AGT AGC CTC AGT GTG GCC CCA TGG TGT GAC TGC AGC AAC        191
Ile Asp Ser Ser Ser Leu Ser Val Ala Pro Trp Cys Asp Cys Ser Asn
         50                  55                  60

AGT GGG AAC GAC CTA GAA GAG TGC TTG AAA TTT TTG AAT TTC TTC AAG        239
Ser Gly Asn Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe Phe Lys
     65                  70                  75

GAC AAT ACA TGT CTT AAA AAT GCA ATT CAA GCC TTT GGC AAT GGC TCC        287
Asp Asn Thr Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser
 80                  85                  90                  95

GAT GTG ACC GTG TGG CAG CCA GCC TTC CCA GTA CAG ACC ACC ACT GCC        335
Asp Val Thr Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr Thr Ala
                100                 105                 110

ACT ACC ACC ACT GCC CTC CGG GTT AAG AAC AAG CCC CTG GGG CCA GCA        383
Thr Thr Thr Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly Pro Ala
            115                 120                 125

GGG TCT GAG AAT GAA ATT CCC ACT CAT GTT TTG CCA CCG TGT GCA AAT        431
Gly Ser Glu Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn
        130                 135                 140

TTA CAG GCA CAG AAG CTG AAA TCC AAT GTG TCG GGC AAT ACA CAC CTC        479
Leu Gln Ala Gln Lys Leu Lys Ser Asn Val Ser Gly Asn Thr His Leu
    145                 150                 155

TGT ATT TCC AAT GGT AAT TAT GAA AAA GAA GGT CTC GGT GCT TCC AGC        527
Cys Ile Ser Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser
160                 165                 170                 175

CAC ATA ACC ACA AAA TCA ATG GCT GCT CCT CCA AGC TGT GGT CTG AGC        575
His Ile Thr Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Gly Leu Ser
                180                 185                 190

CCA CTG CTG GTC CTG GTG GTA ACC GCT CTG TCC ACC CTA TTA TCT TTA        623
Pro Leu Leu Val Leu Val Val Thr Ala Leu Ser Thr Leu Leu Ser Leu
            195                 200                 205

ACA GAA A                                                              630
Thr Glu
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 209 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
Ile Cys Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys Gln Pro Glu
 1               5                  10                  15

Ser Arg Ser Val Ser Ser Cys Leu Lys Glu Asn Tyr Ala Asp Cys Leu
            20                  25                  30

Leu Ala Tyr Ser Gly Leu Ile Gly Thr Val Met Thr Pro Asn Tyr Ile
        35                  40                  45

Asp Ser Ser Ser Leu Ser Val Ala Pro Trp Cys Asp Cys Ser Asn Ser
    50                  55                  60

Gly Asn Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp
65                  70                  75                  80

Asn Thr Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp
                85                  90                  95

Val Thr Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr Thr Ala Thr
            100                 105                 110

Thr Thr Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly
        115                 120                 125

Ser Glu Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu
    130                 135                 140

Gln Ala Gln Lys Leu Lys Ser Asn Val Ser Gly Asn Thr His Leu Cys
145                 150                 155                 160

Ile Ser Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser His
                165                 170                 175

Ile Thr Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro
            180                 185                 190

Leu Leu Val Leu Val Val Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr
        195                 200                 205

Glu
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1075 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS

(B) LOCATION: 2..445

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..1075
(D) OTHER INFORMATION: /note= "1 to 1075 is 1255 to 2330 of Figure 5 Hsgr-2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
T GGG AAC GAC CTA GAA GAG TGC TTG AAA TTT TTG AAT TTC TTC AAG          46
  Gly Asn Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe Phe Lys
    1               5                  10                  15

GAC AAT ACA TGT CTT AAA AAT GCA ATT CAA GCC TTT GGC AAT GGC TCC        94
Asp Asn Thr Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser
                20                  25                  30

GAT GTG ACC GTG TGG CAG CCA GCC TTC CCA GTA CAG ACC ACC ACT GCC       142
Asp Val Thr Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr Thr Ala
            35                  40                  45

ACT ACC ACC ACT GCC CTC CGG GTT AAG AAC AAG CCC CTG GGG CCA GCA       190
Thr Thr Thr Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly Pro Ala
        50                  55                  60

GGG TCT GAG AAT GAA ATT CCC ACT CAT GTT TTG CCA CCG TGT GCA AAT       238
Gly Ser Glu Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn
    65                  70                  75

TTA CAG GCA CAG AAG CTG AAA TCC AAT GTG TCG GGC AAT ACA CAC CTC       286
Leu Gln Ala Gln Lys Leu Lys Ser Asn Val Ser Gly Asn Thr His Leu
80                  85                  90                  95

TGT ATT TCC AAT GGT AAT TAT GAA AAA GAA GGT CTC GGT GCT TCC AGC       334
Cys Ile Ser Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser
                100                 105                 110

CAC ATA ACC ACA AAA TCA ATG GCT GCT CCT CCA AGC TGT GGT CTG AGC       382
His Ile Thr Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Gly Leu Ser
            115                 120                 125

CCA CTG CTG GTC CTG GTG GTA ACC GCT CTG TCC ACC CTA TTA TCT TTA       430
Pro Leu Leu Val Leu Val Val Thr Ala Leu Ser Thr Leu Leu Ser Leu
        130                 135                 140

ACA GAA ACA TCA TAG CTGCATTAAA AAAATACAAT ATGGACATGT AAAAAGACAA       485
Thr Glu Thr Ser  *
    145

AAACCAAGTT ATCTGTTTCC TGTTCTCTTG TATAGCTGAA ATTCCAGTTT AGGAGCTCAG     545

TTGAGAAACA GTTCCATTCA ACTGGAACAT TTTTTTTTTT CCTTTTAAGA AAGCTTCTTG     605

TGATCCTTCG GGGCTTCTGT GAAAAACCTG ATGCAGTGCT CCATCCAAAC TCAGAAGGCT     665

TTGGGATATG CTGTATTTTA AAGGGACAGT TTGTAACTTG GGCTGTAAAG CAAACTGGGG     725

CTGTGTTTTC GATGATGATG ATCATCATGA TCATGATNNN NNNNNNNNNN NNNNNNNNNN     785

NNNNNNNNNN NNNNNNGATT TTAACAGTTT TACTTCTGGC CTTTCCTAGC TAGAGAAGGA     845

GTTAATATTT CTAAGGTAAC TCCCATATCT CCTTTAATGA CATTGATTTC TAATGATATA     905
```

AATTTCAGCC TACATTGATG CCAAGCTTTT TTGCCACAAA GAAGATTCTT ACCAAGAGTG 965

GGCTTTGTGG AAACAGCTGG TACTGATGTT CACCTTTATA TATGTACTAG CATTTTCCAC 1025

GCTGATGTTT ATGTACTGTA AACAGTTCTG CACTCTTGTA CAAAAGAAAA 1075

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 148 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gly Asn Asp Leu Glu Glu Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp
1 5 10 15

Asn Thr Cys Leu Lys Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp
20 25 30

Val Thr Val Trp Gln Pro Ala Phe Pro Val Gln Thr Thr Thr Ala Thr
35 40 45

Thr Thr Thr Ala Leu Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly
50 55 60

Ser Glu Asn Glu Ile Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu
65 70 75 80

Gln Ala Gln Lys Leu Lys Ser Asn Val Ser Gly Asn Thr His Leu Cys
85 90 95

Ile Ser Asn Gly Asn Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser His
100 105 110

Ile Thr Thr Lys Ser Met Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro
115 120 125

Leu Leu Val Leu Val Val Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr
130 135 140

Glu Thr Ser *
145

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1059 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..428

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..1059
(D) OTHER INFORMATION: /note= "1 to 1059 is 1272 to 2330 of Figure 5 Hsgr-9"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
AG TGC TTG AAA TTT TTG AAT TTC TTC AAG GAC AAT ACA TGT CTT AAA          47
   Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn Thr Cys Leu Lys
    1               5                  10                  15

AAT GCA ATT CAA GCC TTT GGC AAT GGC TCC GAT GTG ACC GTG TGG CAG          95
Asn Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp Val Thr Val Trp Gln
                20                  25                  30

CCA GCC TTC CCA GTA CAG ACC ACC ACT GCC ACT ACC ACC ACT GCC CTC         143
Pro Ala Phe Pro Val Gln Thr Thr Thr Ala Thr Thr Thr Thr Ala Leu
            35                  40                  45

CGG GTT AAG AAC AAG CCC CTG GGG CCA GCA GGG TCT GAG AAT GAA ATT         191
Arg Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser Glu Asn Glu Ile
        50                  55                  60

CCC ACT CAT GTT TTG CCA CCG TGT GCA AAT TTA CAG GCA CAG AAG CTG         239
Pro Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln Ala Gln Lys Leu
    65                  70                  75

AAA TCC AAT GTG TCG GGC AAT ACA CAC CTC TGT ATT TCC AAT GGT AAT         287
Lys Ser Asn Val Ser Gly Asn Thr His Leu Cys Ile Ser Asn Gly Asn
80                  85                  90                  95

TAT GAA AAA GAA GGT CTC GGT GCT TCC AGC CAC ATA ACC ACA AAA TCA         335
Tyr Glu Lys Glu Gly Leu Gly Ala Ser Ser His Ile Thr Thr Lys Ser
                100                 105                 110

ATG GCT GCT CCT CCA AGC TGT GGT CTG AGC CCA CTG CTG GTC CTG GTG         383
Met Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro Leu Leu Val Leu Val
            115                 120                 125

GTA ACC GCT CTG TCC ACC CTA TTA TCT TTA ACA GAA ACA TCA TAG             428
Val Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr Glu Thr Ser  *
        130                 135                 140

CTGCATTAAA AAAATACAAT ATGGACATGT AAAAAGACAA AAACCAAGTT ATCTGTTTCC      488

TGTTCTCTTG TATAGCTGAA ATTCCAGTTT AGGAGCTCAG TTGAGAAACA GTTCCATTCA      548

ACTGGAACAT TTTTTTTTTT TCCTTTTAAG AAAGCTTCTT GTGATCCTTT GGGGCTTCTG      608
```

TGAAAAACCT GATGCAGTGC TCCATCCAAA CTCAGAAGGC TTTGGGATAT GCTGTATTTT 668

AAAGGGACAG TTTGTAACTT GGGCTGTAAA GCAAACTGGG GCTGTGTTTT CGATGATGAT 728

GATGATCATG ATGATGATCA TCATGATCAT GATGATGATC ATCATGATCA TGATGATGAT 788

TTTAACAGTT TTACTTCTGG CCTTTCCTAG CTAGAGAAGG AGTTAATATT TCTAAGGTAA 848

CTCCCATATC TCCTTTAATG ACATTGATTT CTAATGATAT AAATTTCAGC CTACATTGAT 908

GCCAAGCTTT TTTGCCACAA AGAAGATTCT TACCAAGAGT GGGCTTTGTG GAAACAGCTG 968

GTACTGATGT TCACCTTTAT ATATGTACTA GCATTTTCCA CGCTGATGTT TATGTACTGT 1028

AAACAGTTCT GCACTCTTGT ACAAAAGAAA A 1059

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 142 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Cys Leu Lys Phe Leu Asn Phe Phe Lys Asp Asn Thr Cys Leu Lys Asn
1 5 10 15

Ala Ile Gln Ala Phe Gly Asn Gly Ser Asp Val Thr Val Trp Gln Pro
20 25 30

Ala Phe Pro Val Gln Thr Thr Thr Ala Thr Thr Thr Thr Ala Leu Arg
35 40 45

Val Lys Asn Lys Pro Leu Gly Pro Ala Gly Ser Glu Asn Glu Ile Pro
50 55 60

Thr His Val Leu Pro Pro Cys Ala Asn Leu Gln Ala Gln Lys Leu Lys
65 70 75 80

Ser Asn Val Ser Gly Asn Thr His Leu Cys Ile Ser Asn Gly Asn Tyr
85 90 95

Glu Lys Glu Gly Leu Gly Ala Ser Ser His Ile Thr Thr Lys Ser Met
100 105 110

Ala Ala Pro Pro Ser Cys Gly Leu Ser Pro Leu Leu Val Leu Val Val
115 120 125

Thr Ala Leu Ser Thr Leu Leu Ser Leu Thr Glu Thr Ser *
130 135 140

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
Gln Ser Cys Ser Thr Lys Tyr Arg Thr Leu
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
Cys Lys Arg Gly Met Lys Lys Glu Lys Asn
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
Leu Leu Glu Asp Ser Pro Tyr Glu Pro Val
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```
Cys Ser Tyr Glu Glu Arg Glu Arg Pro Asn
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```
Pro Ala Pro Pro Val Gln Thr Thr Thr Ala Thr Thr Thr Thr
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CTGTTTGAAT TTGCAGGACT C 21

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CTCCTCTCTA AGCTTCTAAC CACAGCTTGG AGGAGC 36

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTCCTCTCTA AGCTTCTATG GGCTCAGACC ACAGCTT 37

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CTCCTCTCTA AGCTTCTACT TGTCATCGTC GTCCTTGTAG TCACCACAGC TTGGAGGAGC 60

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CTCCTCTCTA AGCTTCTACT TGTCATCGTC GTCCTTGTAG TCTGGCTCAG ACCACAGCTT 60